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Kisspeptin Synchronizes Preovulatory Surges in Cyclical Ewes and Causes Ovulation in Seasonally Acyclic Ewes

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We determined whether kisspeptin could be used to manipulate the gonadotropin axis and ovulation in sheep. First, a series of experiments was performed to determine the gonadotropic responses to different modes and doses of kisspeptin administration during the anestrous season using estradiol-treated ovariectomized ewes. We found that: 1) injections (iv) of doses as low as 6 nmol human C-terminal Kiss1 decapeptide elevate plasma LH and FSH levels, 2) murine C-terminal Kiss1 decapeptide was equipotent to human C-terminal Kiss1 decapeptide in terms of the release of LH or FSH, and 3) constant iv infusion of kisspeptin induced a sustained release of LH and FSH over a number of hours. During the breeding season and in progesterone-synchronized cyclical ewes, constant iv infusion of murine C-terminal Kiss1 decapeptide-10 (0.48 μmol/h over 8 h) was administered 30 h after withdrawal of a progesterone priming period, and surge responses in LH occurred within 2 h. Thus, the treatment synchronized preovulatory LH surges, whereas the surges in vehicle-infused controls were later and more widely dispersed. During the anestrous season, we conducted experiments to determine whether kisspeptin treatment could cause ovulation. Infusion (iv) of 12.4 nmol/h kisspeptin for either 30 or 48 h caused ovulation in more than 80% of kisspeptin-treated animals, whereas less than 20% of control animals ovulated. Our results indicate that systemic delivery of kisspeptin provides new strategies for the manipulation of the gonadotropin secretion and can cause ovulation in noncyclical females. (*Endocrinology* 148: 5258–5267, 2007)

EARLY STUDIES ON the regulation of the hypothalamo-pituitary-gonadotropic axis emphasized the pivotal role of pulsatile GnRH release from hypothalamic neurons for the secretion of the gonadotropes LH and FSH by pituitary, which is essential for gonadal function (for review, see Ref. 1). Nevertheless, the fine control of the pulsatile secretion of GnRH remains poorly defined. For example, during the follicular phase of the ovarian cycle in female mammals, estradiol activates a positive feedback effect that leads to a preovulatory GnRH surge [rat (2), ewe (3, 4), and monkey (5)], but the neuronal mechanisms that underlie this phenomenon are not well understood.

A major advance in this field was made in 2003, with the discovery that the Kiss1/GPR54 system operates in the mammalian brain and regulates the function of GnRH cells. The link between Kiss1/GPR54 and the reproductive system was made in two independent studies reporting that isolated hypogonadotropic hypogonadism in the human is associated with a genetic defect of the *GPR54* gene (6, 7), and that a loss-of-function mutation of *GPR54* gene in mice leads to a deficiency in sexual maturation and infertility (7). The ligand of the GPR54 receptor was first identified as a metastasis suppressor molecule named metastin but was later named kisspeptin (8–10). *Kiss1* is a gene encoding a 145-amino acid peptide that is further processed to generate biologically active peptides of various lengths of 10–54 amino acids named kisspeptins. Kisspeptins bind to GPR54 (11) and display similar high-affinity binding in heterologous cell systems (9).

The discovery that functional GPR54 is critical for reproductive function led to widespread interest in the role of kisspeptins and its receptor. Over the last 4 yr, numerous studies have documented the extraordinary potency of kisspeptins as stimulators of gonadotropin release in numerous species, including the rat, mouse, sheep, monkey, and human (12–16). In rodents, kisspeptins are several orders of magnitude more potent than any other factors recognized as secretagogues for GnRH/LH (13). However, to date, comprehensive dose-response and time-course analysis of the LH releasing effect of peripherally administered kisspeptin has been reported in only one study in rats (17). Given the extraordinary potency of kisspeptins to cause GnRH release in sheep when administered centrally (14), we have undertaken a careful analysis of the pattern of LH and FSH release in response to systemic kisspeptin administration in the ewe. This was done because there are potential applications of this molecule in the control of reproduction. We examined the response to different doses and modes of administration of kisspeptins in ovariectomized (OVX) estradiol-treated ewes during the anestrous season, a model of low basal GnRH and gonadotropin release. The goal was to identify a protocol that would cause LH and FSH secretion similar to that observed

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Abbreviations: AUC, Area under the curve; CIDR, intravaginal controlled delivery device; CL, corpora lutea; CSF, cerebrospinal fluid; hKp10, human kisspeptin-10; ID, inner diameter; mKp10, murine kisspeptin-10; OVX, ovariectomized.

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during a preovulatory gonadotropin surge. Thereafter, the protocol was tested in ewes during the breeding season, with the aim of inducing well-timed and synchronized ovulation. Finally, as we have shown recently that expression of Kiss1 mRNA is reduced during seasonal anestrus and up-regulated at the onset of the breeding season in the ewe (18), we hypothesized that reproductive function could be stimulated in seasonally acyclic ewes by treatment with kisspeptin. Two experiments were conducted in different hemispheres to determine if ovulation could be reliably induced by kisspeptin treatment.

Materials and Methods

Animals

Experiments in France were conducted on Ile de France x Romanov ewes during the breeding season and Ile de France ewes in the anestrous season. The animals (2–3 yr old and weighing 50–60 kg) were maintained under normal husbandry at the Institut National de la Recherche Agronomique research station (Nouzilly), and all experimental procedures were performed in accordance with local animal usage regulation (authorization no. A 38801, French Ministry of Agriculture). The experiment in Australia used East Friesian crossbred ewes of similar weight (76 kg) and age (3–4 yr) that were maintained under natural conditions on a commercial farm. The Australian experiment was performed according to the National Health and Medical Research Council/Commonwealth Scientific and Industrial Research Organization/Australian Animal Code of Practice for the Care and Use of Animals for Experimental Purposes, and the off-site procedure was approved by the Monash University, School of Biomedical Sciences Animal Ethics Committee.

Kisspeptins

Peptides YNWNSFGLRF-NH2 [human kisspeptin-10 (hKp10)] and YNWNSFGLRY-NH2 [murine kisspeptin-10 (mKp10)], corresponding respectively to the human C-terminal Kiss1 decapeptide (112–121-NH2) or murine C-terminal Kiss1 decapeptide (110–119)-NH2 were synthesized by NeoMPS, Strasbourg, France. These sequences are identical to the predicted C-terminal regions of human and ovine metastin (GenBank accession nos. AY117143 and DQ059506, respectively).

Experimental design

Experiments 1–4 were performed during the anestrous season using OVX ewes treated by a sc SILASTIC brand implant (1 cm; Dow Corning Corp., Midland, MI) loaded with estradiol to reduce endogenous GnRH secretion (19). This treatment has reduced LH pulse frequency from 1–0.04 pulses/h in OVX Ile de France ewes (20).

Experiment 1. Dose response to hKp10. The effects of a range of doses of hKp10 upon the dynamic profiles of LH and FSH secretion were evaluated in seven OVX ewes. Jugular venous catheters [inner diameter (ID) 1.0, length 52 mm, Intraflon 2; Vygon, Ecouen, France] were used for hKp10 administration and blood sampling. Each animal was treated each day (over 4 d) with an iv injection of one of the doses of hKp10 (100, 25, 6.2, and 1.6 nmol/injection), given at 1000 h, in a crossover design. Blood samples were taken 60 and 30 min before injection, and 15, 30, 60, 90, 120, 180, 240, 300, and 360 min after injection of the peptide and plasma LH and FSH levels were measured.

Experiment 2. Comparison of response to hKp10 and mKp10. In the course of our study, the sequence of the ovine *kiss1* gene was reported, and the sheep C-terminal Kiss1 decapeptide was found to be identical to murine C-terminal Kiss1 decapeptide (mKp10). Therefore, the relative potencies of mKp10 and hKp10 were compared. Eight estrogen-treated OVX ewes were administered a single iv dose (6.2 nmol) of either hKp10 or mKp10 on sequential days, in a crossover design. Blood samples were taken every 15 min over a 210-min period from 45 min before administration of the peptide and plasma LH and FSH levels were measured.

Experiment 3. Cerebrospinal fluid (CSF) levels of GnRH in response to iv administration of kisspeptin. Experiments 1 and 2 revealed that hKp10 and mKp10 administration induced rapid and short-lived stimulation of LH secretion. To ascertain whether this was due to the release of GnRH, the effects of repeated iv injections of an effective dose of mKp10 (6.2 nmol) on the levels of GnRH into CSF were measured. Six estrogen-treated OVX ewes were prepared with guide cannulae placed into the third ventricles as previously described (21). Two weeks later, a SILASTIC brand catheter was inserted through the guide cannulae into the third ventricles, and CSF was collected every 15 min over 10 h (flow rate 30 μl/min) using a peristaltic pump (Minipuls 2; Gilson, Villier-le-Bel, France), as previously described. Blood samples were collected from the jugular venous catheters simultaneous with each CSF sample, and bolus iv injections of 6.2 nmol mKp10 (n = 6) were given at hourly intervals. GnRH levels in CSF and LH and FSH levels in plasma were measured.

Experiment 4. Sustained responses to kisspeptin. The results of experiment 3 suggested a rapid clearance of mKp10 when administered iv because LH levels increased and then declined rapidly after each injection of peptide. To obtain a more sustained release of gonadotropins, we used two different protocols of peptide administration and examined responses in five estrogen-treated OVX ewes. The two trials were performed in the same animals 15 d apart. First, a sc injection of 150 nmol mKp10 was given, and blood samples were taken every 15 min for 6 h, from 1 h before injection. In the second trial, the same dose was administered as a constant iv infusion (25 nmol/h; flow rate 500 μl/h) over 6 h using portable syringe pumps (Graseby Medical, Watford, Hertfordshire, UK) connected to jugular catheters by polyethylene tubing (ID 1.0, length 50 cm; Vygon). Blood samples were taken using a second jugular venous catheter every 15 min from 1 h before the start of the infusion until 11 h afterwards. Plasma LH and FSH levels were measured.

Experiment 5. Synchronization of the preovulatory LH surge in cyclical ewes during the breeding season. The estrous cycles of normal ewes (n = 18) were synchronized by administration of prostaglandin (0.1 ml Estrumate, cloprostenol; Intervet, Angers, France), followed by insertion of an intravaginal progesterone controlled delivery device (CIDR) (InterAg, Hamilton, New Zealand) for 14 d. Animals were then housed in individual pens with access to food and water, and the progesterone CIDRs were removed. Commencing 30 h later, mKp10 (3.84 μmol; n = 9) or vehicle (n = 9) was infused iv over 8 h (0.48 μmol/h; flow rate 500 μl/h), using portable syringe pumps (Graseby Medical) connected to jugular venous catheters. The rationale for this higher dose of mKp10 (compared with 0.150 μmol/6 h in experiment 4) was to maximize the likelihood of obtaining an increase in plasma LH levels similar in magnitude to that seen during the endogenous preovulatory LH surge. Blood samples were taken by venipuncture every 2 h between 26 and 64 h after progesterone CIDR removal to measure plasma LH levels and every 12 h for 5 d after the day of infusion to measure progesterone levels. On d 6 after the infusions, the ovaries of the ewes were inspected by laparoscopy, and the number of active corpora lutea (CL) on each ovary was recorded.

Experiment 6. Stimulation of the gonadotropin axis and ovulation by kisspeptin in seasonally acyclic ewes. The first replicate of this experiment was performed in April in France, being the nonbreeding season for sheep in the Northern Hemisphere. After weekly measurement of progesterone levels over 1 month (data not shown), 26 anestrous Ile de France ewes were selected for the study, and two different protocols of peptide administration were used. In one series, ewes were administered mKp10 as bolus iv injections (12.6 nmol every 12 h for 60 h, n = 7) or vehicle (saline) injections (control, n = 7). Blood samples were taken by venipuncture in windows at 0, 24, and 48 h after commencement of bolus iv treatment with mKp10 or vehicle. In each series, blood samples (5 ml) were collected immediately before and at every 15 min after the scheduled injection for 2.5 h. Plasma LH levels were measured.

In a second series of animals, bilateral jugular venous catheters (ID 1.0 mm, Intraflon 2) were inserted for blood sampling and constant iv infusion of either mKp10 (n = 6; 0.6 μmol over 48 h; flow rate 500 μl/h; 12.6 nmol/h) or vehicle (n = 6), using portable syringe pumps (Graseby Medical). In these animals, blood samples were collected every 15 min for 2 h before infusion and for the first 2 h of the infusion period. Blood was also collected midway through the infusion every 15 min for 2 h

(22–24 h) and also every 15 min for the final 2-h infusion (46–48 h). Plasma LH levels were measured.

In both experimental series (pulse treatment or infusion), blood samples were taken to ascertain whether preovulatory like LH surges occurred. These blood samples were taken every 3 h between 27 and 57 h from the start of the mKp10 or vehicle infusions, and between 54 and 84 h from the start of treatment in the animals receiving mKp10 injections. Laparoscopy was performed 6 d after the completion of treatment to determine the presence or absence of CL, indicating ovulation. For one animal receiving mKp10 infusion, the pump malfunctioned, and this animal was removed from the experiment.

This experiment was replicated in with a group of East Friesian sheep during November in Australia. This period is the middle of the nonbreeding season for this breed of sheep in the Southern Hemisphere. There were 10 sheep that were administered mKp10 by iv infusion, and 10 received vehicle (saline) as controls. Ten days before the infusion period, the animals received CIDRs for progesterone treatment, with the intention of increasing the ovulatory response to 100%. At CIDR removal, animals were transferred to group pens in a farm shed and fitted with iv infusion cannulae (SILASTIC brand tubing, ID 1.02 mm, outer diameter 2.16 mm) connected to 20-ml plastic syringes driven by Graseby MS16A infusion pumps (Smith Medical Australasia Pty. Ltd., Gold Coast, Queensland, Australia). The dose of mKp10 was 0.38 μmol over 30 h (12.6 nmol/h at a flow rate of 500 μl/h). Venipuncture blood samples were taken immediately before commencement of infusion, and 2 and 12 h after the start of infusion for the measurement of plasma LH levels. After the infusion, animals were returned to the field, and laparoscopic examinations were performed 5 d later to determine whether ovulation had occurred by observing CL on the ovaries. At this time, and again at 9 and 11 d after the infusion, venipuncture blood samples were taken for the assay of progesterone in plasma to determine whether functional CL had formed. After infusion, two pumps of the kisspeptin-infused group were found to have malfunctioned. By laparoscopic examination, one mKp10-treated animal was found to have significant pelvic inflammation and adhesions. These three animals were removed from the experimental analysis.

Hormone assays

France. Plasma LH concentrations were measured using a previously described RIA (22, 23), with all samples from one experiment being included in a single assay. The assay standard was 1051-CY-LH (equivalent to 0.31 National Institutes of Health LH-S1). The intraassay coefficient of variation averaged 9%, and assay sensitivity was 0.2 ± 0.05 ng/ml (four assays). Plasma FSH levels were measured using the reagents supplied by the National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases (Bethesda, MD). The intraassay coefficient of variation averaged 9.5%, and assay sensitivity was 0.2 ng/ml relative to the standard (oFSH 19-SIAFP RP2). The cross reactivity with ovine LH was 0.6%. Progesterone was measured in a single assay (24) after hexane extraction of 100 μl plasma, with an assay sensitivity of 0.1 ng/ml and an intraassay coefficient of variation of 10%. GnRH concentrations in CSF samples were measured using a previously described RIA (25), with the intraassay coefficient of variation and assay sensitivity being 14% and 1.5 pg/ml, respectively (five assays).

Australia. Plasma LH concentrations were measured in duplicate, using the method of Lee *et al.* (26) with National Institutes of Health-oLH-S18 as standard. Assay results were calculated using the program of Burger *et al.* (27). Assay sensitivity was 0.1 ng/ml, and the intraassay coefficient of variation was less than 10% over the range of 0.6–9.6 ng/ml. Plasma progesterone levels were measured in a single assay based on the method of Deayton *et al.* (28), with a sensitivity of 0.3 ng/ml and intraassay coefficient of variation of 5%.

Data presentation and statistical analysis

Data are presented as the mean (±SEM). Apart from individual time-point determinations, integrated LH and FSH secretory responses were estimated by calculation of the area under the curve (AUC) of the concentration *vs.* time plots above the baseline (mean level preceding the peptide injection). AUC was calculated with the trapezoidal rule over the 120-min period after administration of kisspeptin in experiments 1 and 2. The duration of the kisspeptin-induced LH pulses in experiment 1 was defined as the time interval when LH levels were above the baseline. An LH surge (experiment 5) was defined as a sustained increase in LH concentrations, being twice the mean concentration preceding the administration of mKp10 or vehicle and exceeding 10 ng/ml in at least one sample. Comparisons of AUC for LH and FSH in experiments 1 and 2 as well as the comparison of numbers of CL in experiment 5 were made using nonparametric exact tests with StatXact 5 Software (Cytel Software Corp., Cambridge, MA). The Bonferroni correction was used for *ad hoc* comparisons of the groups 2 × 2. In experiment 1, comparisons of AUC for LH and FSH across the four doses of kisspeptin were performed with a Page test, and comparisons of hKp10 and mKp10, dose, and duration of the kisspeptin-induced LH pulses were made with a permutation test for paired samples. In experiment 2, comparisons of AUC for LH and FSH between the two doses of kisspeptin were performed with a permutation test for paired samples. In experiment 5, a comparison of numbers of CL on the ovaries of the two groups of ewes was made with a permutation test. Mean levels of progesterone (experiment 5) were compared by two-way ANOVA with repeated measures of two factors (time and treatment), and the Student's unpaired *t* test was used as a *post hoc* test. In experiment 6, ovulation data were analyzed by the χ^2 test and are expressed as a percentage of animals ovulating. Variations in plasma LH and progesterone levels were assessed by two-way ANOVA (treatment × time). Where a statistical significant treatment-time interaction was shown, vehicle and kisspeptin treated groups were examined at each time point by the Student's unpaired *t* test. The level of statistical significance was set at 5%.

Results

Experiment 1: dose-response to hKp10

As shown in Fig. 1A, all doses of hKp10 effectively stimulated LH release within 15-min injection, with maximal levels ranging between 2- and 10-fold higher than those

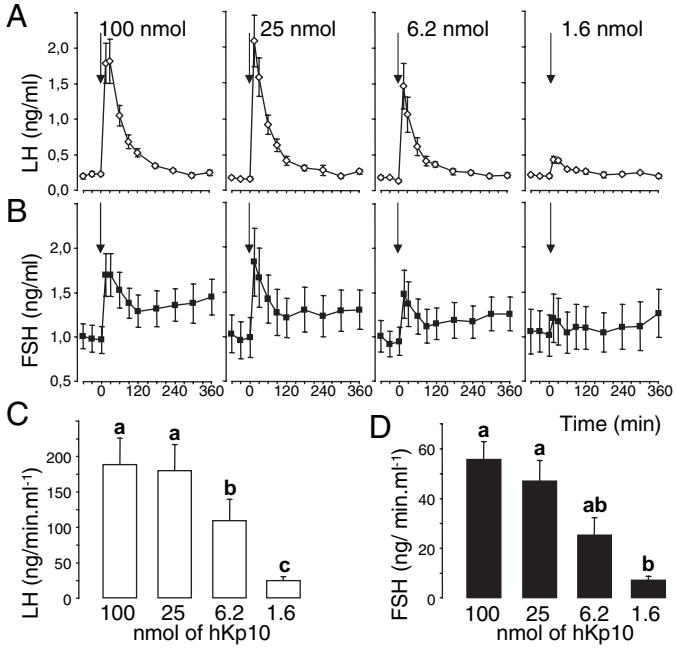

FIG. 1. Effects of iv administration of a single bolus of hKp10 at different doses (100, 25, 6.2, or 1.6 nmol/injection) upon the pattern of LH and FSH secretion (A and B) in OVX ewes (mean ± SEM; n = 7). For each group, *arrows* indicate the time point when iv injection is given. In addition to dynamic profiles, net LH and FSH secretion evoked by the different doses of hKp10 is presented in panels C and D as integrated hormone response (AUC) over the 120-min period after injection of hKp10. Groups with *different superscript letters* are statistically different ($P < 0.0083$).

before injection. The mean amplitude of the LH responses elicited by 1.6 nmol hKp10 was significantly ($P < 0.02$) lower than the amplitude of response to higher doses, and the duration of the elicited pulse of secretion was also shorter ($P < 0.001$); the LH level had returned to preinjection values within 120 min after injection compared with more than 240 min for the three other doses. There was a clear dose effect on the magnitude of the integrated LH secretory response (AUC) to hKp10 ($P < 0.001$; Fig. 1C). AUC in response to 6.2 nmol was lower ($P < 0.01$) than that evoked by 25 and 100 nmol, which were of similar magnitude, but greater than the response induced by 1.6 nmol ($P < 0.05$).

FSH release was also stimulated by administration of hKp10, but the response appeared different from that of LH (Fig. 1B). There was a rapid increase in plasma FSH levels within 15-min injection, but the magnitude of the response was smaller than for LH, with maximal levels being 1.7- to 1.8-fold greater than preinjection values for the two higher doses. Moreover, whereas LH levels decayed after a rapidly attained peak after hKp10 injection, plasma FSH levels were more sustained. Over the 120 min after injection, there was a clear dose effect on the magnitude of the integrated FSH secretory response (AUC) to hKp10 ($P < 0.001$; Fig. 1D). FSH response to 6.2 nmol hKp10 was approximately half that evoked by 25 and 100 nmol but was not different to the response induced by 1.6 nmol peptide.

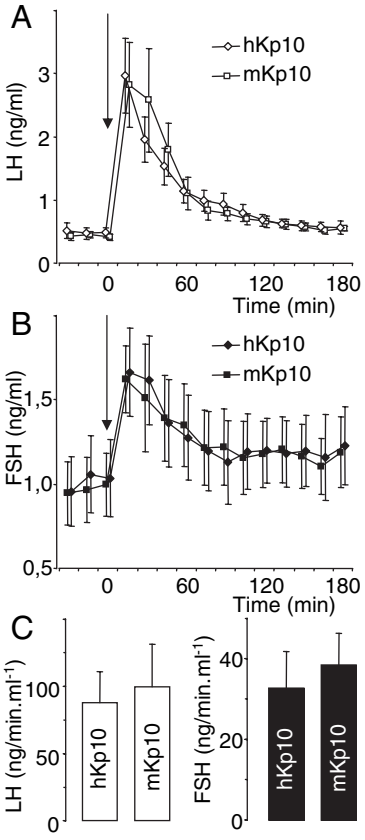

FIG. 2. Comparison of iv administration of a single bolus of hKp10 and mKp10 (6.2 nmol/injection) upon the pattern of LH (A, *open symbols*) and FSH secretion (B, *closed symbols*) in OVX ewes (mean ± SEM, n = 8). *Arrows* indicate the time point when the iv injection is given. C (*lower panel*), The integrated hormone response (AUC) over the 120-min period after injection of hKp10 or mKp10 was not different for LH or FSH.

Experiment 2: comparison of response to hKp10 and mKp10

There was no difference in the dynamic LH (Fig. 2A) and FSH responses (Fig. 2B) to hKp10 and mKp10, with AUC responses being similar for the two peptides (Fig. 2C). For both peptides (hKp10 and mKp10), LH levels returned to baseline within 2-h injection, whereas FSH levels remained above preinjection values until the end of the blood-sampling period.

Experiment 3: CSF levels of GnRH in response to iv administration of kisspeptin

Before iv administration of mKp10, GnRH concentrations into the CSF were in the range of the detection limit of the assay (Fig. 3A), but within 15–30 min of each iv injection of 6.2 nmol mKp10, an increase in the GnRH concentration was measured, with maximal levels approximately 2-fold higher than assay sensitivity. After the last mKp10 injection, GnRH concentrations in CSF declined rapidly, returning to assay sensitivity within 45 min. Each evoked increase in CSF GnRH levels was associated with a coincident increase in plasma LH (Fig. 3B) and FSH (Fig. 3C) levels. As in experiments 1 and 2, the magnitude of the responses for LH appeared larger than for FSH.

Experiment 4: sustained responses to kisspeptin

Figure 4 shows LH and FSH responses to either sc injection of 150 nmol mKp10 or constant iv infusion. The amplitude of the LH response to a single sc dose (150 nmol) of mKp10 was 3.6-fold the baseline ($P < 0.05$; Fig. 4A), and the duration

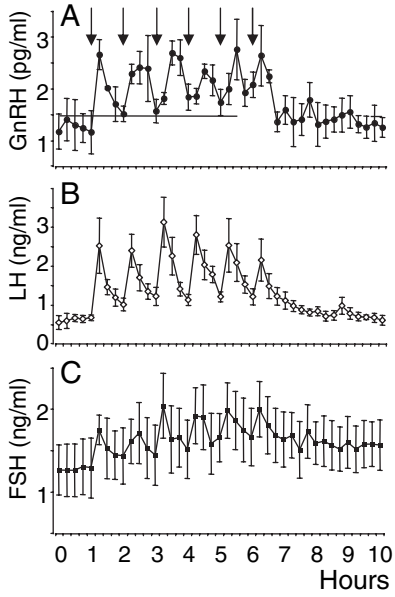

FIG. 3. Effects of iv administration of repeated bolus (n = 6) of mKp10 (6.2 per injection) upon the pattern of GnRH into the CSF (A) and LH and FSH (B and C) in the peripheral circulation in OVX ewes (mean ± SEM, n = 6). The time of each injection is indicated by *arrows*. The *horizontal line* (*upper panel*) indicates the concentration above which a value can be considered significantly different from zero.

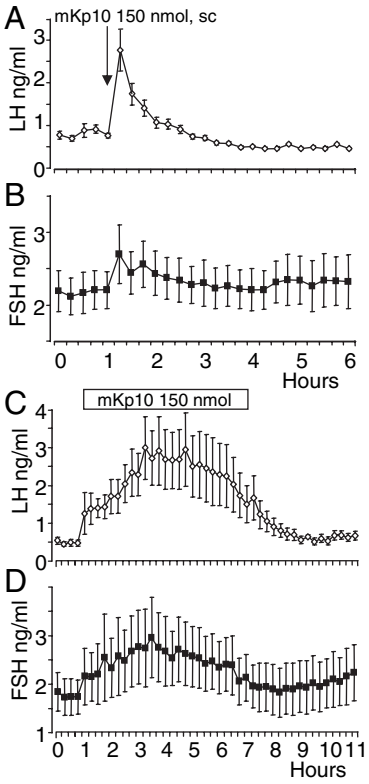

FIG. 4. Effects of a single sc administration of mKp10 (150 nmol; A and B) or a constant infusion of the peptide (150 nmol over 6 h; C and D) upon the pattern of LH and FSH in the peripheral circulation in OVX ewes (mean ± SEM, n = 5). The time of the sc injection is indicated by the *arrows*, whereas the duration of the peptide infusion is represented by the *horizontal bar*.

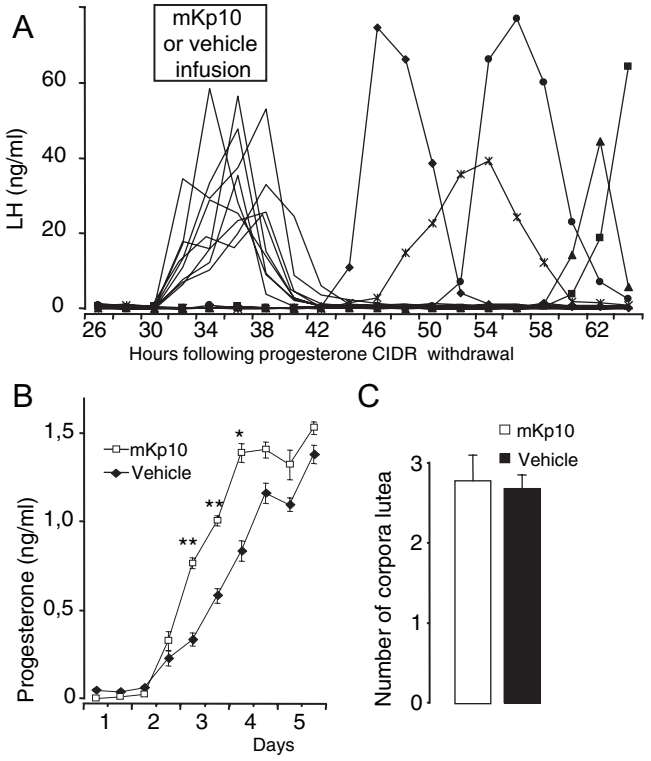

FIG. 5. A, The *solid lines* represent the pattern of LH secretion of the ewes (n = 9) receiving a constant infusion of mKp10 (3.84 μmol for 8 h) given 30 h after progesterone CIDR removal. The *solid lines with the closed symbols* represent the pattern of LH secretion of the ewes (five of nine) receiving the infusion of the vehicle and showing an LH surge during the period of blood sampling. B, Pattern of progesterone secretion over the 5 d after the day of infusion of mKp10 (*open squares*) or the vehicle (*closed lozenge*). C, Mean of CL number (d 6 after infusion) in ewe treated by mKp10 (*open bar*) or vehicle (*closed bar*). Values are the means ± SEM (n = 9). *, $P < 0.05$. **, $P < 0.01$.

of the response was 90 min. FSH levels were also increased after the sc administration of the peptide ($P < 0.05$; Fig. 4B), but the magnitude of the response was smaller than that of LH. Constant iv infusion of the same dose of mKp10 (150 nmol over 6 h) produced a sustained increase in plasma levels of both LH and FSH, with a significant ($P < 0.05$; Fig. 4, C and D) increase being apparent within 15- to 30-min commencement of infusion and a peak response occurring 2–4 h later. Thereafter, during continuous infusion of peptide, a progressive decline of the levels of both hormones occurred.

Experiment 5: synchronization of the preovulatory LH surge in cyclical ewes in the breeding season

After constant iv administration of 3.84 μmol mKp10 over 8 h given 30 h after withdrawal of progesterone priming, LH surges occurred in all animals over a short time frame (Fig. 5A). The onset of surges in the mKp10-treated ewes occurred 32 ± 0 h after progesterone CIDR removal, with peak values ranging from 25.0–58.3 ng/ml. The elevation in LH levels was apparent in the first blood samples taken after the start of the mKp10 infusion, and LH concentrations returned less than 2 ng/ml within 4 h of the end of the infusion in eight of nine ewes (*i.e.* 42 h after progesterone CIDR removal). For one ewe, the period of infusion was extended from 8–10 h, due to a technical problem with the infusion pump, and, therefore, the LH surge was slightly more prolonged. Five of nine animals receiving the vehicle also showed LH surges during the period of sampling, but the onset of these surges was more variable and occurred later (commencing 44–60 h after progesterone CIDR removal) than those obtained with mKp10 infusion. Plasma progesterone levels increased earlier in animals receiving mKp10 (time × treatment; $P < 0.05$), and significant differences in the levels in the treatment and control groups were observed at d 2.5 ($P < 0.01$), 3 ($P < 0.01$), and 3.5 ($P < 0.05$) (Fig. 5B). Examination of ovaries 5 d after administration of mKp10 or vehicle did not show any difference in the mean number of CL in the two groups of ewes (2.8 ± 0.3 *vs.* 2.7 ± 0.2 in mKp10 and the vehicle groups, respectively; Fig. 5C).

Experiment 6: stimulation of the gonadotropin axis and ovulation by kisspeptin in seasonally acyclic ewes

French study. In animals that received repeated injections of mKp10, significant responses were obtained in terms of plasma LH levels; this varied over time with a significant interaction ($P < 0.001$) (Fig. 6A). LH concentrations were significantly elevated after mKp10 treatment (compared with vehicle) when examined at the 0 h ($P < 0.001$), and 24 ($P < 0.01$) and 48 h injection ($P < 0.01$). Although the re-

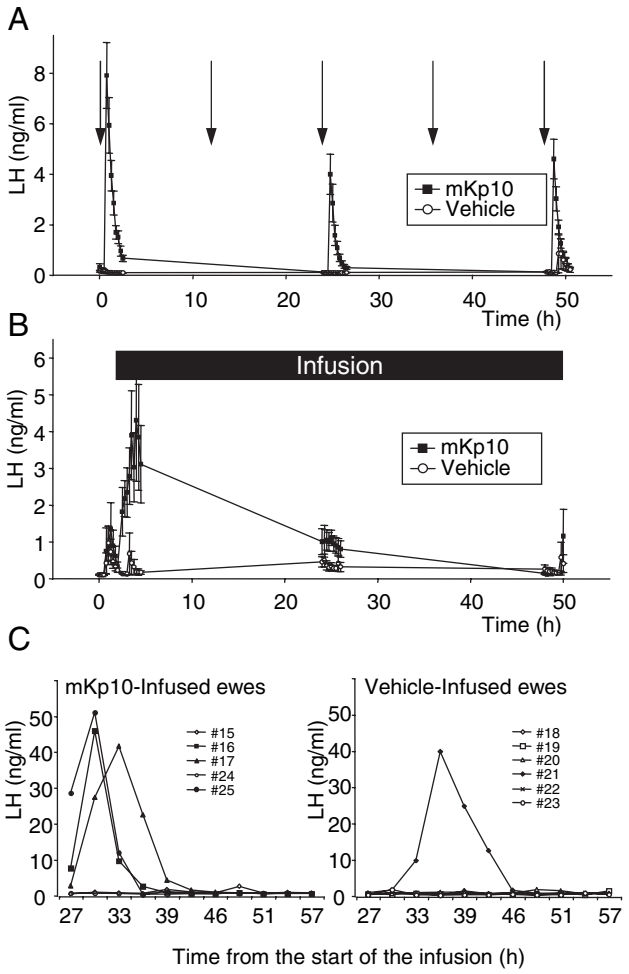

FIG. 6. Plasma LH concentrations (French experiment) in anestrous ewes treated with mKp10 (*closed squares*) or vehicle (*open circles*). Values (mean ± SEM) show the LH response to repeated peripheral bolus injections of mKp10, or vehicle (A), or peripheral infusion of mKp10, or vehicle (B). A, *Arrows* indicate the timing of mKp10 injections. Note that plasma LH levels were measured in samples taken across the 0-, 24-, and 48-h injections. B, The period of infusion is represented by the *horizontal bar*. C, Individual LH profiles of the mKp10 (*left*) and vehicle-infused ewe (*right*) from 27–57 h after the onset of infusion.

sponses at the second and third period tested were lower (significant difference between 0 and 48 h; $P < 0.01$), responsivity was maintained throughout the experimental period. Despite this, blood samples taken every 3 h indicated no instance of a preovulatory surge in these animals (data not shown), and none of the treated animals (zero of seven) ovulated.

For the animals receiving infusions of mKp10, two-way ANOVA showed a significant ($P < 0.001$) effect of treatment (compared with vehicle) on plasma LH concentrations and a significant time-treatment interaction ($P < 0.001$). mKp10 elevated plasma LH levels during the first 2-h infusion ($P < 0.05$). During the 24- to 26-h sampling period, plasma LH levels in the treated animals decreased but remained higher than in vehicle-infused controls ($P < 0.05$). At the 48- to 50-h sampling period, plasma LH levels were similar in mKp10-infused and vehicle-infused animals (Fig. 6B). Additional blood samples taken every 3 h between 27 and 57 h from the onset of the infusion showed that LH surges occurred in three of five mKp10-infused animals and in one of six vehicle-infused animals (Fig. 6C). Based on the presence of CL on the ovaries observed by laparoscopic examination 5 d after treatment, ovulations occurred in four of five Ile de France ewes (80%) receiving mKp10 by constant iv infusion and one of six (17%) vehicle-infused ewes ($P < 0.05$; see Fig. 8A).

Australian study

After 2-h infusion, plasma LH levels were elevated ($P < 0.05$) in the mKp10-treated animals, with no similar effect of vehicle treatment (Fig. 7). After the infusion had terminated, plasma LH concentrations were far more variable with two mKp10-treated ewes, and one vehicle-infused ewe showing elevated levels. Notably, this control animal was the same that was later observed to have ovulated.

After infusion of mKp10, six of seven (86%) treated ewes and one of 10 (10%) control ewes ovulated ($P < 0.01$; Fig. 8B). For these later animals, plasma progesterone levels demonstrated similar results to laparoscopic examination. All ewes that displayed CL (six of seven treated and one of 10 control) had elevated progesterone levels ($P < 0.01$, mKp10; Fig. 8C).

Discussion

We have examined the effects of kisspeptin on GnRH secretion, gonadotropin secretion, and ovulatory responses in the ewe, and the main findings were: 1) iv administration of kisspeptin is a potent stimulus of LH and FSH secretion; 2) this gonadotropin response to kisspeptin is most likely due to evoked GnRH release; 3) constant infusion of kisspeptin induces prolonged LH and FSH release, and produces tightly timed LH surges and ovulation in this species; and 4) low-dose infusion of the peptide during the anestrous season stimulates gonadotropin secretion and induces ovulation in acyclic females.

It is especially notable that a dose as low as 6.2 nmol hKp10 can evoke unambiguous LH and FSH responses after iv injection to ewes. Analysis of the subsequent integrated hormone responses shows that the magnitude of the response to 6.2 nmol is about half that obtained with either 25 or 100 nmol, which induce similar responses. Thus, on the basis of

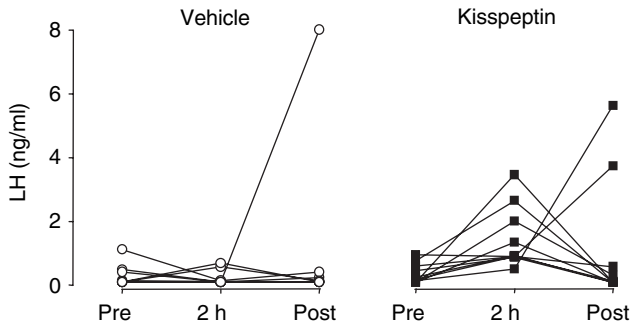

FIG. 7. Plasma LH concentrations (Australian experiment) in anestrous ewes treated with kisspeptin-10 (*closed squares*) or vehicle (*open circles*). Values are from individual ewes immediately before (Pre), 2 h after infusion onset (2 h), and approximately 12 h after the infusion (Post).

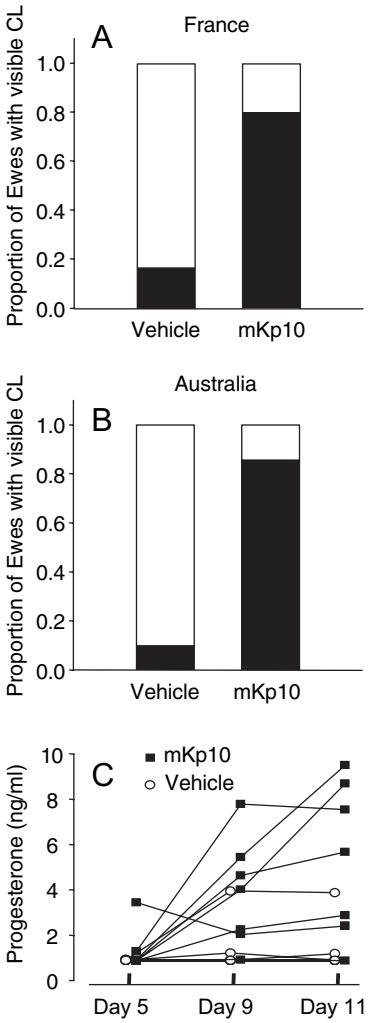

FIG. 8. A and B, The proportion of anestrous ewes that ovulated after peripheral infusion of mKp10 during the nonbreeding season in the Northern Hemisphere (France) and Southern Hemisphere (Australia), respectively. *Closed bars* indicate the proportion of ewes that ovulated, whereas *open bars* indicate the proportion that failed to ovulate. C, Plasma progesterone concentrations in anestrous ewes after peripheral infusion of mKp10. Values are shown for the Australian experiment, and are from individual ewes 5, 9, and 11 d after the infusion of mKp10 (*closed squares*) or vehicle (*open circles*). In all cases, ewes that showed CL via laparoscopic examination, including the one vehicle-treated ewe, had elevated progesterone concentrations at 9 and 11 d.

body weight (~50 kg for the ewes of the present study), a dose of 0.12 nmol/kg is able to cause secretion of LH and FSH. This is remarkably similar to the half-maximal dose of 0.3 nmol/kg reported for rats (17).

Whereas the release of LH is in response to discrete secretory episodes of GnRH secretion (29, 30), the secretory profile of FSH suggests that secretion is "constitutive" (31). Nevertheless, the present study shows a clear secretory response to kisspeptin for both gonadotropins after iv administration. This is consistent with data obtained in rats (12) and monkeys (32), and is most likely due to evoked GnRH secretion, based on our measurements in CSF. Nevertheless, direct pituitary action of kisspeptin cannot be excluded (33). However, as we have shown in sheep, the magnitude of the FSH response is lower than for LH but is more prolonged. After central administration of kisspeptin to rats, FSH release appeared approximately 100-fold less sensitive to the stimulatory effect of the peptide than LH (34). We found that iv injection of 6.2 nmol hKp10 induced about half the maximum response for both gonadotropins. The difference between the two studies may reflect species differences, route of peptide administration (intracerebroventricular *vs.* iv), or physiological status of the animals used.

We found that plasma FSH levels remained elevated for more than 6 h after iv injection of kisspeptin at doses higher than 6.2 nmol, which has implications regarding the mechanism of action of the peptide. The immediate stimulatory effect of the peptide on LH and FSH occurs most likely through a stimulation of GnRH release. Certainly, it has been shown that iv coadministration of a GnRH antagonist blocks kisspeptin-induced LH and FSH release in rats (13, 35) and monkeys (32), and kisspeptin induces GnRH release from *in vitro* hypothalamic explants (36). This suggests that the stimulation of LH and FSH release by kisspeptin depends upon GnRH release, which is consistent with previous results from this laboratory (14) as well as the results of experiment 3 of the present study. Each iv injection of mKp10 caused a transitory elevation of GnRH levels in CSF of the third ventricle. The observation that iv administration causes sustained elevation of plasma FSH levels, with no hint of decline within 6-h hKp10 injection, suggests that there is a secondary mechanism that sustains the FSH response. This may be an indication of a direct pituitary action because kisspeptin can enhance GnRH-stimulated FSH secretion from pituitary tissue, at least in rats, at low concentrations, but not at high concentrations (34). Thus, it is possible that, after the initial GnRH-induced LH and FSH release by hKp10 administration, there is a secondary effect of the peptide at the level of the gonadotrope, to enhance constitutive FSH release, in sheep at least. Additional studies are needed to clarify this point.

We have shown that the potency of human and mouse kisspeptin is similar in terms of the ability to cause LH and FSH release (experiment 2). A recent publication reporting the structure-activity relationships of kisspeptin molecules shows that substitution of the C-terminal phenylalanine by tyrosine does not affect either binding affinity or agonist potency of kisspeptin (37), which is consistent with our data. Therefore, both molecules can be alternatively used to stimulate gonadotropins in this species.

Repeated iv administration of mKp10 induced a sustained train of LH and FSH secretory responses in estradiol-treated OVX ewes (experiment 3), which is similar to results obtained in agonadal monkeys (32) and rats (17). The clearly pulsatile pattern of GnRH that the repeated iv injections produce in CSF further suggests a short-lived response and a short half-life of kisspeptin. The plasma half-life of a longer kisspeptin-54 molecule is reported to be 28 min in humans (16), with indications that shorter fragments (kisspeptin 14 and kisspeptin 10) have shorter half-lives (32). This is consistent with the short-lived GnRH response and the response of the pituitary gland gonadotropes in the present studies. The decapeptide form of kisspeptin is the shortest sequence of the full-length parent peptide that is biologically active,

with the C-terminal sequence being essential for receptor activation (8). As a consequence of this short half-life of kisspeptins, it is possible to obtain repeated responses to bolus injections of the peptide, without an increasing baseline in LH levels. However, this is not the case for FSH due to the longer-lasting FSH response. Subcutaneous administration of the peptide (experiment 4) was no more efficient than the iv route to induce gonadotropin release, indicating that the molecule is cleared or inactivated when administered by this route as well (38). A continuous increase in plasma gonadotropin level over several hours could only be achieved when mKp10 was given as a constant infusion, but the amplitude of the gonadotropin "surge" response was lower with this paradigm than seen during the natural preovulatory gonadotropin surge. Furthermore, plasma gonadotropin levels began to decrease before the end of the peptide infusion. This phenomenon has also been observed in primates given infusions of kisspeptin and was attributed to desensitization of the GPR54 receptor with continuous exposure to ligand (39). Endogenous GnRH secretion is profoundly suppressed in OVX anestrous ewes by estradiol treatment due to enhanced sensitivity to the negative feedback effect of the steroid at this time of the circannual reproductive cycle (19, 40). In this model, GnRH treatment is required to restore pituitary LH content (41) as well as pituitary GnRH receptor levels (42). Accordingly, the limited amplitude of the gonadotropin response to continuous administration of mKp10 in this model must be considered in relation to the physiological status of the animals.

In experiment 5, we tested the hypothesis that constant iv infusion of mKp10 would synchronize the timing of ovulation in progesterone-primed ewes. Treatment clearly achieved synchronization of LH surges. It is possible that these surges were due to the release of surge-like levels of GnRH in animals that were given mKp10. This could be regarded as a pharmacological intervention that presents the surge signal to an estrogen-primed pituitary, or it could be early induction of the positive feedback event. We cannot distinguish between these two possibilities, but in any event, the infusion of kisspeptin can clearly advance the surge and synchronize ovulation in progesterone-primed ewes. It must be pointed out that the responses in these mKp10-treated animals were greater than those seen in the estrogen-treated OVX ewes treated during the anestrous season, probably for the reasons indicated previously. Nevertheless, it should be appreciated that we used a much higher dose (3.84 μmol over 8 h) of kisspeptin in experiment 5 to maximize the chance of achieving the desired result. The amplitudes of these mKp10 induced-LH surges were lower than those occurring in the controls, although this difference did not attain statistical significance. Because the response achieved in the cyclical ewes was most likely due to a combination of stimulated GnRH secretion and increased pituitary sensitivity to GnRH that occurs around the time of the onset of the LH surge in the ewe (42), it may be hypothesized that the priming of the pituitaries of the kisspeptin-infused ewes was not optimal for these advanced synchronized LH surges. Nevertheless, the amount of LH released during the surges in each mKp10-treated animal was sufficient to induce ovulation, as evidenced by the observed presence of active CL on the ovaries and the clear increase in plasma progesterone concentrations during the days after treatment. This certainly offers some potential use for kisspeptin or analogs to manipulate the timing of ovulation, but more experiments are needed to determine whether these slightly advanced ovulations affect the quality of oocyte.

The final experiment, conducted in two hemispheres and with different breeds of sheep, indicates that low-dose infusion of kisspeptin during the anestrous season stimulates the gonadotropin axis and induces ovulation in acyclic females. Kisspeptin treatment has also stimulated ovulation in juvenile rats (13). However, it must be pointed out that these animals were primed with pregnant mare's serum gonadotropin, which stimulates gonadal function and causes ovulation in sheep and goats (43). Our data show that kisspeptin infusion can cause ovulation in anestrous ewes, whether or not they are primed with progesterone. Progesterone treatment *per se* did not cause ovulation in a significant number of control animals that were subsequently infused with vehicle (Australian experiment). This offers potential for a treatment of acyclicity because the kisspeptin treatment is effective in a continuous delivery mode, allowing for the possibility of using implants to induce ovulation.

Whereas an initial LH response is seen within hours of commencement of infusion, this appears to "activate" the system with ovulatory surges occurring approximately 30 h from the start of treatment. This provides insight into the mechanism whereby this treatment is effective, showing that "normal" LH surges are elicited with a time delay consistent with estrogen positive feedback. Accordingly, we conclude that the delayed surge secretion of LH, and not the initial LH response at the commencement of infusion, is the stimulus for ovulation. This is quite different from the immediate and highly synchronized kisspeptin-induced LH surge observed in the late follicular phase of the cyclical ewes (experiment 5). This synchronization was effected in animals in which preovulatory surges were imminent, and the mechanism of the effect leading to the immediate surge response is likely to be different from that that we describe in anestrous ewes with a surge generating mechanism already primed by estradiol (44), leading to increased sensitivity to GnRH (45). In our studies during the anestrous season, infusion of kisspeptin causes long-lasting elevation in plasma FSH levels (experiments 1–4). Because this gonadotropin is responsible for the aromatization of testosterone to estrogen in the ovary (46, 47), it may be that this is the operative mechanism in the seasonally anestrous ewe treated with kisspeptin. Estrogen treatment of anestrous ewes causes LH surges within a delay of approximately 12 h (48, 49). In further support of this hypothesis, the interval between the beginning of the kisspeptin infusion and the onset of the LH surge in Ile de France ewes during the nonbreeding season (~30 h) is in the same range as the interval between the progesterone CIDR removal and onset of the LH surge during the breeding season (50). On this basis, we conclude that the estrogen-feedback mechanism is activated in the anestrous ewes, and it is this that causes a delayed positive feedback and surge secretion of GnRH/LH.

To our knowledge there are no previous studies that have examined the effects of continuous infusion of kisspeptin in

females of any species. In adult male rats, chronic sc administration of hKp10 (50 nmol/d) caused degeneration of the testes after 13-d treatment (38). In this instance, a stimulatory effect of kisspeptin on LH levels was observed only during the first day of treatment. No effect was seen thereafter, suggesting down-regulation of the response to kisspeptin during continuous administration, possibly by GPR54 desensitization. In another study, chronic iv infusion of 100 μg/h hKp10 to juvenile male rhesus monkeys stimulated LH above the levels of vehicle-treated controls for approximately 24 h, and no effect on LH was seen for 4 d thereafter (39). Again, it was suggested that constant infusion caused desensitization of GPR54 because injection of the GnRH secretagogue N-methyl-DL-aspartic acid or GnRH, but not kisspeptin, was able to stimulate LH secretion on d-4 infusion. Thus, the integrity of GnRH neurons was intact, but the system had failed to respond to kisspeptin (39). Similarly, we showed that the LH response is not maintained with continuous mKp10 infusion, so GPR54 down-regulation might have occurred. Despite this, the acute response to infusion of mKp10 is such that preovulatory like LH surges occurred and caused ovulation in the majority of animals. We conclude that despite the lack of continued elevation in LH levels, the administration of kisspeptin to acyclic females is sufficient to activate the hypothalamo-pituitary axis and cause ovulation. Further work is required to determine the minimal period of treatment with kisspeptin that is required for this response.

Whereas infusion of mKp10 clearly caused ovulation in the majority of animals, treatment with multiple iv bolus injections had no effect. This was surprising because the bolus injections elicited robust responses in terms of plasma LH concentrations. The treatment regimen was designed to provide stimulation of gonadotropin secretion that would, in turn, stimulate the ovaries to provide an increase in estrogen that led to a positive feedback event and ovulation. Clearly, although the treatments effectively elicited LH responses, these were not sufficient to activate the ovaries. It is possible that more frequent pulsatile treatment or variation in dosage could be effective, but given the efficacy of the constant infusion, further investigation was considered unwarranted.

In summary, our study shows that kisspeptin is a potent stimulator of gonadotropin secretion in sheep. The demonstration that continuous infusion can synchronize LH surges in progesterone-primed cyclical ewes and cause ovulation in seasonally acyclic ewes paves the way for rational therapeutic use of kisspeptin, or kisspeptin analogs, in the pharmacological manipulation of the gonadotropin axis in mammals.

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