Kisspeptin Is Essential for the Full Preovulatory LH Surge and Stimulates GnRH Release from the Isolated Ovine Median Eminence

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Kisspeptins are the product of the *Kiss1* gene and potently stimulate GnRH secretion. In sheep, *Kiss1* mRNA-expressing cells are found in the arcuate nucleus (ARC) and dorsal-lateral preoptic area and both appear to mediate the positive feedback effect of estradiol to generate the preovulatory GnRH/LH surge. To determine the role of kisspeptin in transmitting estrogen-positive feedback in the hypothalamus, we administered the kisspeptin antagonist p-271 to ewes subjected to an estradiol benzoate-induced LH surge. Kisspeptin antagonist treatment significantly attenuated these LH surges. We further examined the response to kisspeptin treatment prior to the LH surge. Kisspeptin significantly stimulated GnRH secretion into the hypophysial portal system, but the response to kisspeptin was similar in luteal and late-follicular phase ewes. *Kiss1r* mRNA expression in GnRH neurons was also similar across the estrous cycle. To examine alternative pathways for kisspeptin stimulation of GnRH neurons, we examined the origin of kisspeptin neuronal fibers in the external zone of the median eminence (ME) using neuronal tracing and immunohistochemical techniques. ARC populations of kisspeptin neurons project fibers to the ME. Finally, we showed kisspeptin stimulates GnRH release from ovine ME-cultured explants. This suggests direct kisspeptin to GnRH terminal-to-terminal communication within the ME. Overall, these data indicate an essential role for kisspeptin in receiving stimulatory estrogen signals and generating the full positive feedback GnRH/LH surge. Kisspeptin neurons of the ARC project to the external zone of the ME and kisspeptin acts upon the GnRH fibers at this level. **(*Endocrinology* 152: 1001–1012, 2011)**

Kisspeptin cells in the brain have proven to be a conduit for gonadal steroid feedback control of GnRH secretion (1, 2). Thus, kisspeptin appears important for both the negative- and positive-feedback signals of estradiol (E2). In sheep, kisspeptin cells are located in the dorsolateral preoptic area (POA) and arcuate nucleus (ARC) (3). Kisspeptin cells in the ovine ARC appear to be involved in positive-feedback regulation of GnRH (4) and also appear to be important for negative-feedback regulation of GnRH secretion (5). Further studies in sheep (6, 7) and primates (8) show kisspeptin cells in the POA may also play a role in generating the estrogen positive-feedback preovulatory GnRH/LH surge.

Disabling mutations or genetic deletions of the kisspeptin receptor gene (*Kiss1r*) result in the failure to reach puberty (9, 10), but there is disagreement as to whether mice with null mutations in *Kiss1r* are able to mount an estrogen positive-feedback response and LH surge (11,

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.

doi: 10.1210/en.2010-1225 Received October 21, 2010. Accepted December 13, 2010.
First Published Online January 14, 2011

Abbreviations: aCSF, Artificial cerebrospinal fluid; ARC, arcuate nucleus; AUC, area under the curve; BDA, biotinylated dextran amine; CV, coefficient of variation; DIG, digoxigenin; E2, estradiol; EB, E2 benzoate; LV, lateral ventricle; ME, median eminence; NKB, neurokinin B; OVX, ovariectomized; POA, preoptic area.

12). E2 treatment induced LH surges in *Kiss1r*$^{-/-}$ mice in one study (12) but did not in another (11). Because both studies involved congenital absence of *Kiss1r*, it becomes important to block endogenous kisspeptin signaling in nongenetically modified animals and determine whether positive-feedback responses persist. We have characterized a kisspeptin antagonist (13) that prevents the LH surge in rats (14). This protocol of chronic administration, however, did not determine whether the antagonist effect was due to down-regulation of the reproductive axis, preventing an E2 rise, or whether it blocked the positive-feedback effect of E2 at the kisspeptin/GnRH interface. Therefore, our first aim was to determine the role of kisspeptin in facilitating the E2 positive-feedback effect. We examined the effect of central kisspeptin antagonist treatment on the E2-induced preovulatory-like LH surge in anestrous ewes.

Recently the LH surge has been associated with an increase in the LH response to kisspeptin in humans and sheep (15, 16), indicating the surge may be generated by increased kisspeptin output and sensitivity. It remains unclear whether this is a direct reflection of increased kisspeptin stimulation of GnRH secretion into hypophysial portal blood. We measured GnRH levels in portal blood in response to kisspeptin treatment in ovary-intact ewes during the luteal and late-follicular phases of the estrous cycle (immediately before the GnRH/LH surge). Moreover, we determined the expression of Kiss1r on GnRH neurons in luteal and late-follicular animals.

Despite the fact that kisspeptin stimulation of GnRH secretion is important for the generation of the LH surge, the neuroanatomical connection between kisspeptin and GnRH is yet to be fully understood. In the ovine brain, POA kisspeptin cells provide direct input to GnRH neurons in the ventral POA, whereas kisspeptin cells in the ARC do not (17). Similar data are evident in mice (18). Therefore, alternative pathways from ARC kisspeptin cells to GnRH neurons may exist. In sheep and primates, kisspeptin neurons project to the median eminence (ME), in which varicose fibers come into close apposition to GnRH fibers (2, 19) for possible axoaxonic regulation of GnRH secretion, which has been demonstrated in mice (20). We determined the origin of kisspeptin ME fibers and examined the effect of kisspeptin on GnRH release from the isolated ovine ME to ascertain functional significance.

Materials and Methods

Animals

Corriedale ewes of similar age (5–6 yr) and weight were maintained at the Monash University Sheep Facility (Werribee, Victoria, Australia) under natural conditions of ambient photoperiod and environmental temperature. All experiments were carried out according to the National Health and Medical Research Council/Commonwealth Scientific and Industrial Research Organisation/Australian Animal Commission Code of Practice for the Care and Use of Animals for Experimental Purposes and were approved by the Monash University, School of Biomedical Sciences Animal Ethics Committee.

Peptides

Kisspeptin peptide YNWNSFGLRY-NH2 corresponding to the murine C-terminal *Kiss1* decapeptide (110-119)-NH2 was obtained from Phoenix Pharmaceuticals Ltd. (Belmont, CA). This sequence is identical to the C-terminal region of ovine kisspeptin (GenBank accession no. DQ059506) and has been characterized in our previous experiments (21). Kisspeptin antagonist (p-271) was synthesized by EZBiolab Inc. (Carmel, IN). The peptide sequence of p-271 comprises that of p-234 [ac-(D-A)NWNGFG(D-W)RF-NH2] (13) with the NH_2 terminal addition of a seven-amino acid cationic cell-penetrating peptide penetratin RRMKWKK– via an additional tyrosine residue. This peptide was recently tested in rats and was shown to be an effective kisspeptin antagonist (14).

Experimental design

Experiment 1: effect of kisspeptin antagonist on LH in ovariectomized (OVX) ewes

To first examine the role of kisspeptin in the tonic pulsatile release of LH, we administered the kisspeptin antagonist p-271 to OVX ewes. Ewes were bilaterally OVX at least 1 month before any experimental manipulation. Permanent indwelling lateral ventricle (LV) cannulae were then implanted as described previously (22). Approximately 2 wk after LV surgery, one external jugular vein was cannulated for blood sampling and animals were housed in single pens. The following day, infusion pumps (Graseby MS16A; Graseby Medical Ltd., Gold Coast, Australia) were connected to LV cannulae and blood samples (5 ml) collected every 10 min for 7 h. After 3 h, ewes received either kisspeptin antagonist (1 h continuous infusion of 300 μg/h, with an initial 200 μg loading dose; n = 5) or vehicle [artificial cerebrospinal fluid (aCSF); n = 5] into the LV (200 μl/h) (13). The dose of antagonist was determined by preliminary experiments in OVX ewes (data not shown). After infusion, LV lines remained in place as blood sampling continued. Plasma was harvested immediately and frozen at −20 C until assayed.

Experiment 2: effect of kisspeptin antagonist on the LH surge in estrogen-treated anestrous ewes

To determine the critical role of kisspeptin signaling in mediating E2-positive feedback and generating the LH surge, we administered the kisspeptin antagonist p-271 to anestrous ewes under an E2-induced GnRH/LH surge model (23). LV and jugular vein cannulae were implanted (as above) in ewes during the Southern Hemisphere anestrous season (September). All ewes received an im injection of 50 μg E2 benzoate (EB; Intervet, New South Wales, Australia) in 1 ml peanut oil. Blood sampling commenced 9 h later, and samples (5 ml) were taken every 10 min for 9 h and then every 30 min for a further 12 h; one final blood sample was then taken 34 h after EB treatment. At 12 h after EB injection, ewes received LV infusions (200 μl/h) of kisspeptin antagonist (8 h continuous infusion 300 μg/h, with an initial 200

μg loading dose; n = 6) or vehicle (aCSF; n = 6). This time frame for treatment was chosen to begin at least 2 h before the predicted LH surge, which is known to occur between 14 and 18 h after EB treatment (23). Plasma was harvested immediately and frozen at −20 C until assayed.

Experiment 3: LH and GnRH responses to kisspeptin in luteal and late-follicular phase ewes

In this experiment we sought to determine whether the LH surge is characterized by a shift in the GnRH response to kisspeptin. During the breeding season, ewes were prepared for hypophysial portal and jugular vein sampling as previously described (23). Estrous cycles were synchronized by an im injection of the synthetic luteolysin, cloprostenol (Estrumate, 125 μg; Pitman-Moore, Sydney, New South Wales, Australia) as previously described (16), and ewes were treated during the late-follicular phase and the luteal phase. Blood samples (5 ml) were collected every 10 min for 4 h. After 2 h, kisspeptin (50 μg, diluted in 4 ml physiological saline) or vehicle (n = 5–6 per group) was administered via the jugular cannula. The dose was based on our previous data (16, 21). Portal blood samples were collected into tubes containing 100 μl of 5 mM bacitracin (Sigma, St. Louis, MO) and held on ice with corresponding jugular samples; additional samples of jugular blood were collected into bacitracin and served as peripheral controls for the GnRH assay. Plasma was harvested within 10 min of collection and stored at −20 C for RIA. At the completion of the portal sampling, the sheep were killed and the ovaries inspected to confirm reproductive phase.

Experiment 4: Kiss1r mRNA expression on GnRH neurons in luteal and late-follicular phase ewes

If changes in kisspeptin response are determined in experiment 3, these may be facilitated by changes in *Kiss1r* expression on GnRH neurons at the time of the LH surge. Ewes in the luteal and late-follicular phase (n = 4–5 per group, determined as above) were killed by an iv overdose of sodium pentobarbital (Lethabarb; Virbarc, Peakhurst, Australia). Heads were perfused and the hypothalamus dissected as previously described (6). Coronal sections (40 μm) were cut on a cryostat and placed into cryoprotectant (30% ethylene glycol, 20% glycerol in sodium phosphate buffer with 2% paraformaldehyde) and stored at −20 C until used for *in situ* hybridization.

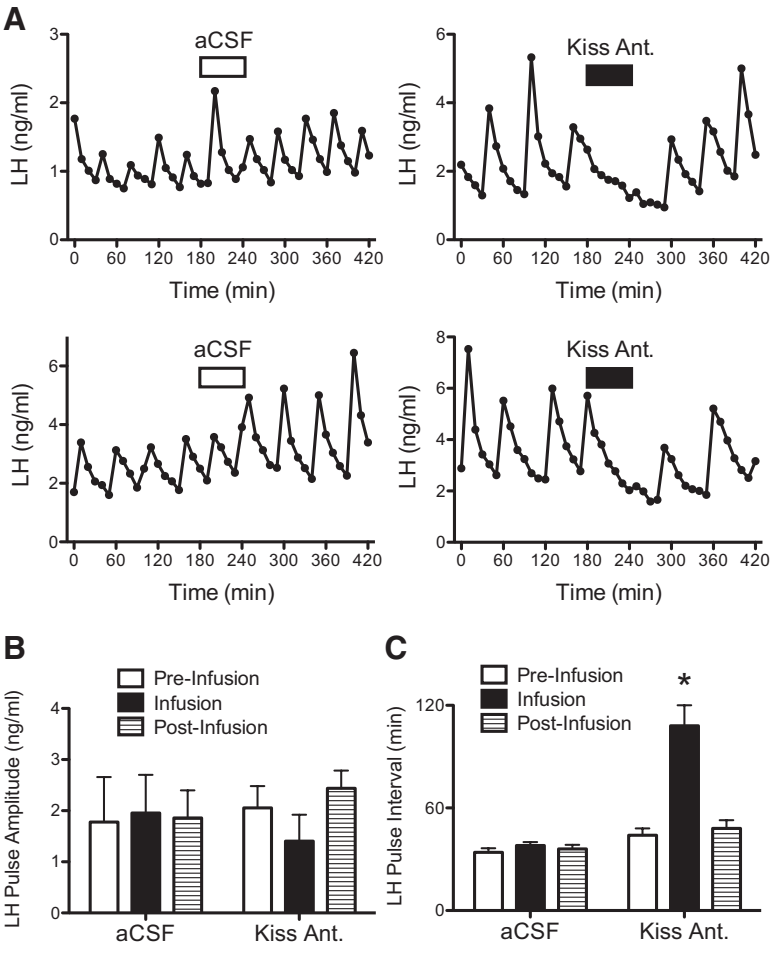

FIG. 1. Central infusion of kisspeptin antagonist (Kiss Ant.) inhibits the secretory pulses of LH in OVX ewes. A, Concentrations of LH are shown in two representative animals treated with kisspeptin antagonist (*closed bar*) or aCSF (*open bar*). B and C, LH pulse amplitude (B) and pulse interval (C) during 2-h time periods before infusion, during infusion, and immediately after infusion, and after infusion (n = 5 per group). A significantly prolonged pulse interval during kisspeptin antagonist infusion was detected. Data are the mean ± SEM. *, $P < 0.05$.

Experiment 5: anatomical origin of kisspeptin fibers in the ME

This study further examined the nature of neuroanatomical connection between kisspeptin and GnRH kisspeptin fibers in the ME. Because virtually all kisspeptin cells in the ARC coexpress neurokinin B (NKB) (24) and kisspeptin cells in the POA do not, we used this as a marker for kisspeptin fiber origin in the ME. OVX ewes (n = 4) were killed as above and brains processed for double-label immunohistochemistry. Hypothalamic blocks were carefully prepared so that the entire ME was intact in coronal sections. To confirm that kisspeptin cells of the ARC region project to the external zone of the ME, we injected 70 nl of the anterograde neurotracer biotinylated dextran amine (BDA) into the ARC of OVX ewes (n = 4), using methods previously described (25). The animals were euthanized (as above) after 3 wk and hypothalamic blocks were processed for double-label immunohistochemistry.

Experiment 6: Kiss1r *mRNA expression on Kiss1 neurons*

Because a proportion of kisspeptin neurons in the POA received kisspeptin fibers appositions, we sought to determine whether kisspeptin cells possess *Kiss1r*. OVX ewes (n = 4) were killed and brains processed for double-label *in situ* hybridization as above in experiment 4.

Experiment 7: GnRH response to kisspeptin stimulation in cultured ME explants

To determine physiological significance for the kisspeptin fibers in the ME, we examined the local effect of kisspeptin on the GnRH secretion from the ME. ME culture experiments were performed similar to those previously described for the mouse mediobasal hypothalamus (20). OVX ewes were killed as

above and the ME immediately dissected and placed in individual wells of a 24-well plate with 1 ml DMEM (Thermo Electron Corp., Melbourne, Australia) prewarmed to 37 C. The plate was transferred to a shaker in a culture incubator at 37 C with 95% O_2-5% CO_2 atmosphere. After 1 h preincubation, media were replaced with 1 ml fresh media containing kisspeptin (10, 100, 1000 nM) or vehicle treatment (n = 5–7 per group). After 1 h, the medium was harvested, stabilized with 100 μl bacitracin (Sigma), and stored at −20 C. To confirm the viability of the tissue after the experiment, the ME was exposed to 60 nM KCl (in DMEM) for 1 h. The ME cultures showing no GnRH response to KCl were removed from the study. DMEM with no ME was simultaneously incubated under the same conditions to serve as a negative control. One ME-intact hypothalamus and one hypothalamus after ME dissection were additionally postfixed and processed for hematoxylin and eosin staining to detail the ME dissection.

Double-label *in situ* hybridization

The cDNA templates for a *Kiss1r*, *GnRH*, and *Kiss1* riboprobes were generated as previously described (6). The *Kiss1r*-specific template spanned bases 635–714 of the ovine cDNA sequence (GenBank accession no. EU272411). The antisense ovine *Kiss1r* riboprobe was transcribed from cDNA template with T7 polymerase (Promega, Madison, WI) and ^{35}S-uridine 5-triphosphate (GE Healthcare Life Sciences, Little Chalfont, UK) using a standard transcription protocol. The *GnRH*-specific template spanned bases 18–169 of the ovine partial cDNA sequence (GenBank accession no. U02517). The *Kiss1*-specific template spanned bases 1–357 of the ovine partial cDNA sequence (GenBank accession no. DQ059506). Digoxigenin (DIG)-labeled antisense *GnRH* and *Kiss1* riboprobes were transcribed with a MEGAscript T7 transcription kit (Ambion, Austin, TX) and DIG labeling mix (Roche, Indianapolis, IN) according to the manufacturer's protocol.

For *Kiss1r*/*GnRH* analysis, three sections through the POA from each ewe were chosen; for *Kiss1r*/*Kiss1* analysis, three sections through the POA and three through the ARC were chosen. Sections were chosen, slides processed, and double-label *in situ* hybridization performed as previously described (6). Sections were hybridized with both ^{35}S-labeled *Kiss1r* riboprobe (5×10^6 cpm/ml) and DIG-labeled *GnRH* or *Kiss1* riboprobe (1:400, determined empirically) at 54 C overnight. *GnRH*- or *Kiss1* mRNA-containing cells were visualized with bright-field microscopy and *Kiss1r* mRNA was assessed under dark-field illumination. For *Kiss1r*/*Kiss1 in situ* hybridization, *Kiss1r*/*GnRH*-hybridized slides were included as a positive control for *Kiss1r*, and for all probes, no signal was observed after the application of radiolabeled sense probe (data not shown). Grain-counting software (ImagePro plus; Media Cybernetics, Silver Spring, MD) was used to count the silver grains (*Kiss1r* mRNA) over each *GnRH* or *Kiss1* cell. Signal to background ratios were calculated and cells were considered double labeled with a ratio of 3 or greater. For each ewe, the percentage of *GnRH* or *Kiss1* cells with *Kiss1r* mRNA was averaged for each animal and a group mean (±SEM) was then calculated.

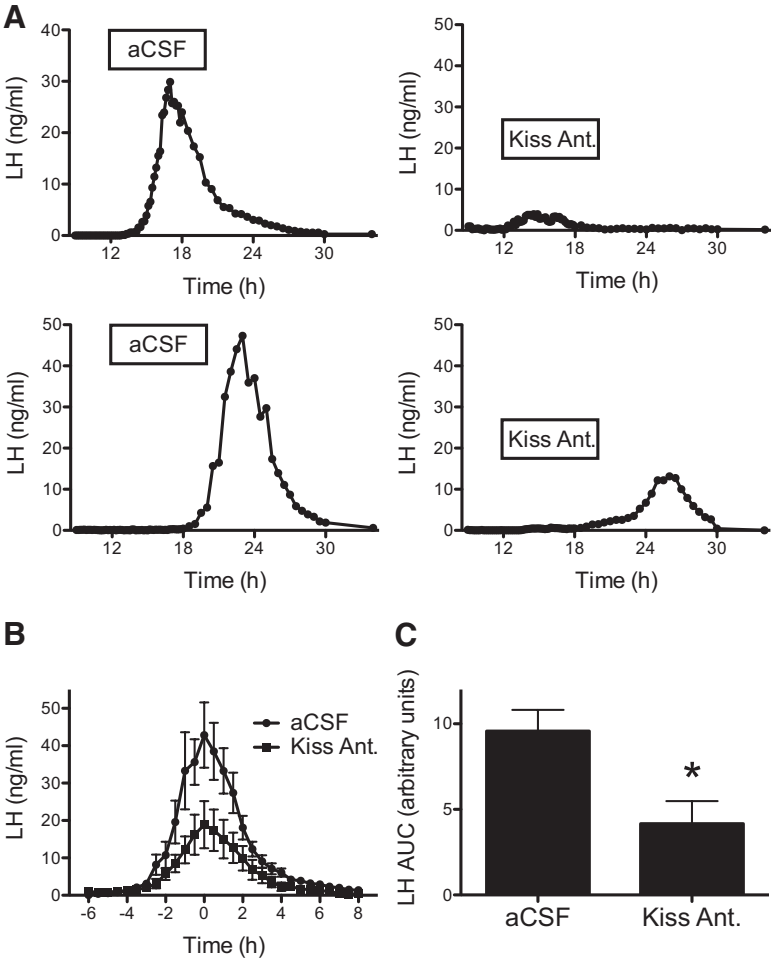

FIG. 2. Central infusion of kisspeptin antagonist (Kiss Ant.) inhibits the estrogen induced preovulatory-like LH surge. A, Concentrations of LH are shown in four representative animals treated with kisspeptin antagonist (*closed bar*) or aCSF (*open bar*, n = 6 per group). The x-axis shows time (hours) from estrogen treatment. B, Mean (± SEM) LH surges in ewes treated with kisspeptin antagonist or aCSF. The x-axis is time (hours) from the peak of the LH surge. C, AUC analysis revealed a significant decrease in the LH surge in ewes treated with kisspeptin antagonist. Data are the ± SEM. *, $P < 0.05$.

Double-label immunohistochemistry

Sections through the ARC/mediobasal hypothalamus and POA were chosen (6) from each ewe for analysis. Double-label immunohistochemistry for NKB and kisspeptin was performed as previously described (24, 26). Guinea pig antisera against NKB (27) and rabbit antisera against kisspeptin (3) were provided by Professor Philippe Ciofi (Neurocenter Magendie, Bordeaux, France) and Professor Alain Caraty (Universite′ Tours, Nouzilly, France), respectively. For kisspeptin and BDA detection, the kisspeptin antibody was used as above. BDA was detected by streptavidin-Texas red (1:500; Vector, Burlingame, CA). Fluorescent images of the hypothalamus and in particular the ME were merged to determine whether anterograde tracer and kisspeptin were present in neuronal fibers in the external neurosecretory zone. Putative contacts of kisspeptin/NKB fibers on kisspeptin cells in the ARC and POA were examined with Z-stack microscopy using a Zeiss Apotome microscope (Carl Zeiss, Inc., North Ryde, Sydney, Australia) as previously de-

scribed (28, 29). To confirm the structural interactions between kisspeptin and GnRH fibers at the ME in the sheep, kisspeptin and GnRH immunohistochemistry was performed. GnRH was detected with a monoclonal antibody against GnRH (1:2000, HU11B; Urbanski, Oregon Regional Primate Research, Beaverton, OR) and antimouse aminomethylcoumarin acetate (1:500; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA).

Hormone RIA

Plasma LH concentrations were measured in duplicate, using the method of Lee *et al.* (30). Assay sensitivity was 0.2 ng/ml and the intraassay coefficient of variation (CV) was less than 10% over the range of 0.4–10.7 ng/ml in experiment 1, 0.8–19.3 ng/ml in experiment 2, and 0.6–11.3 ng/ml in experiment 3. The interassay CV was 9%.

GnRH was measured by the method of Jonas *et al.* (31). Portal plasma (1 ml) was extracted with acidified methanol, evaporated dry, and then reconstituted in 1 ml assay buffer (recovery of GnRH was determined using the addition of cold hormone to plasma). Culture media were not extracted. All samples were assayed in duplicate. Assay sensitivity was 0.5 pg/ml and the intraassay CV was less than 10% between 10 and 712 pg/ml. The interassay CV was 4%.

Data analysis

In experiment *1*, LH pulse analysis was performed based on the method described for GnRH (23). The effect of kisspeptin antagonist treatment was determined by comparing LH pulse amplitude and pulse interval in 2-h time periods before (1–3 h), during, and immediately after infusion (3–5 h), and after (5–7 h) treatment. The period duration (minutes) was divided by the number of LH pulses detect in the period to give the appropriate interval. In experiments 2 and 3, area under the curve (AUC) for LH was determined using the trapezoidal rule, allowing integration for unequally spaced x values in Sigma Plot 9.0 (Point Richmond, CA). LH surges were taken to have begun when a clearly evident monophasic rise in plasma LH levels occurred. AUC for kisspeptin response in experiment 3 was derived from baseline-corrected response curves for 2 h after treatment (LH) or 1 h after treatment (GnRH) as previously described (16). In experiment 5, the percentage of kisspeptin staining overlapping NKB staining was determined by Manders' coefficients using JACoP (32) with ImageJ 1.43u (National Institutes of Health, Bethesda, MD). All grouped data are presented as the mean ± SEM. LH pulse amplitude, pulse interval, and kisspeptin response AUC were assessed by two-way ANOVA. Mean LH surge and LH/GnRH kisspeptin response curves were assessed by one-way and two-way repeated-measures ANOVA. LH surge AUC, *Kiss1r* mRNA data, and ME culture data were assessed by one-way ANOVA using Tukey's multiple comparison *post hoc* test. Differences were considered significant when $P < 0.05$.

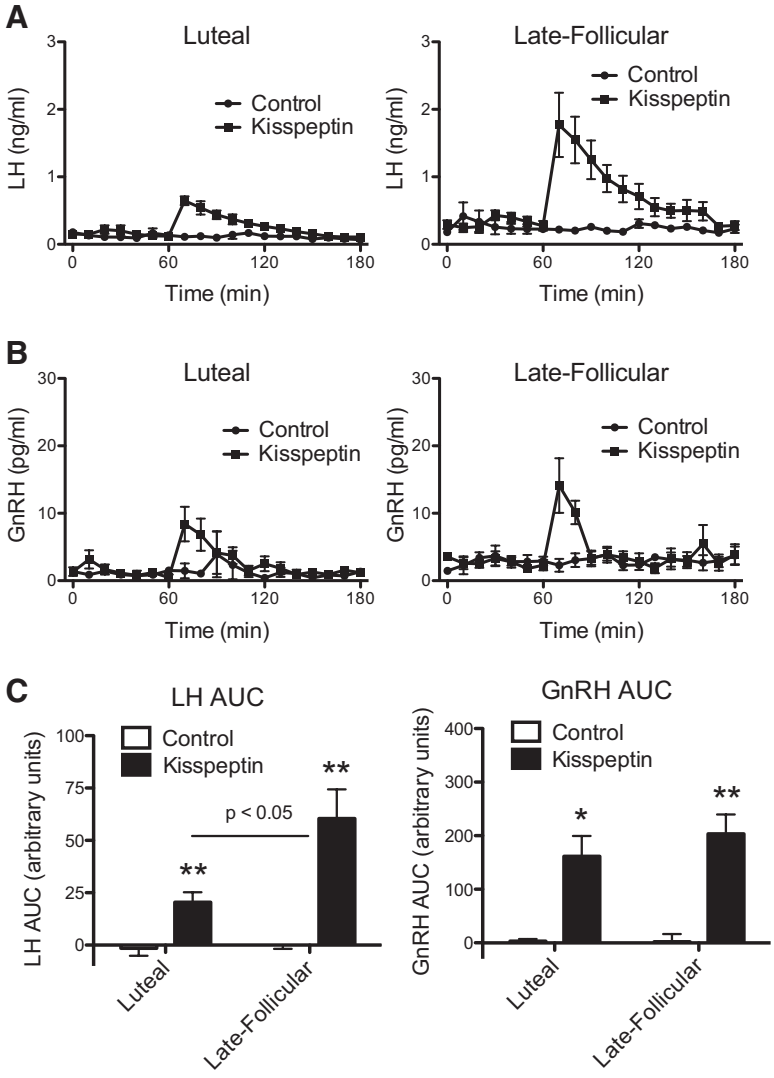

FIG. 3. LH (A) and GnRH (B) responses to kisspeptin in luteal and late-follicular phase ewes. Kisspeptin treatment (50 μg, iv) or vehicle control (saline) was delivered at 60 min. C, AUC for LH and GnRH responses to kisspeptin in luteal and late-follicular phase ewes. The AUC was calculated from response curves (60–180 min for LH and 60–120 min for GnRH). Repeated-measures ANOVA revealed a significant effect of kisspeptin treatment for both LH and GnRH: *, $P < 0.05$; **, $P < 0.01$. Analysis also revealed a significant effect of phase (with a significant interaction) for LH data only. For LH responses among kisspeptin-treated groups, the *P* value is shown. All data are the mean ± SEM (n = 5–6 per group).

Results

Central infusion of kisspeptin antagonist inhibits LH pulses

The aim of this experiment was to examine the role of kisspeptin in the tonic pulsatile release of LH. p-271 showed clear antagonist actions on LH levels in OVX ewes. Pulsatile secretory episodes of LH were evident in aCSF control ewes and treated ewes before and after kisspeptin antagonist treatment (Fig. 1A). No detectable LH pulses were noted during the 1-h kisspeptin antagonist infusion period but were detected in the 1 h afterward. These LH pulses were similar to those in control animals and were reflected in the LH pulse amplitude data

(Fig. 1B). LH pulse interval was significantly greater in kisspeptin antagonist-treated ewes during and immediately after the infusion ($P < 0.05$, Fig. 1C).

Central infusion of kisspeptin antagonist inhibits the EB-stimulated LH surge in anestrous ewes

To determine the importance of kisspeptin signaling in transmitting the estrogen-positive feedback signal to induce the LH surge, an intracerebroventricular infusion of kisspeptin antagonist was applied to ewes under an EB-induced LH surge protocol. The LH profile is shown for four representative ewes in Fig. 2A. LH surges occurred in all EB/aCSF-treated control ewes, the onset occurring 16.2 ± 1.5 h (range 14–22 h) after EB injection. The magnitude of the EB-induced LH surges was diminished by central p-271 treatment (Fig. 2A), with no effect on surge onset (15.6 ± 1.7 h; range 12–19 h after EB injection). The mean LH surge and area under the LH surge curve were significantly lower (AUC 56% lower, both $P < 0.05$, Fig. 2, B and C) in kisspeptin antagonist-treated ewes.

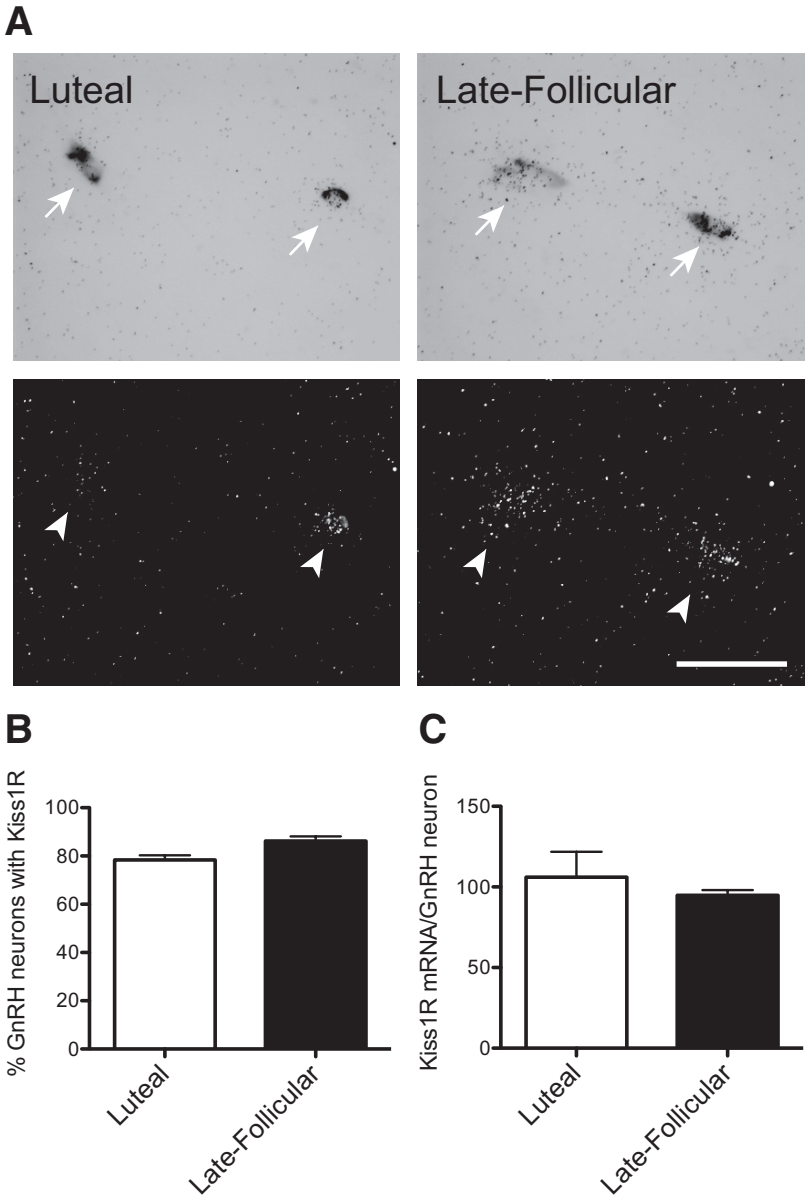

FIG. 4. Expression of *Kiss1r* mRNA in GnRH neurons in luteal and late-follicular phase ewes. A, Representative bright-field photomicrographs show cells expressing GnRH mRNA (*arrows*, *top panel*, visualized with nitroblue tetrazolium substrate) and corresponding dark-field photomicrographs (*lower panel*) show coexpression of *Kiss1r* mRNA (*arrowheads,* clusters of *silver grains*; note: silver grains can also be seen in bright-field illumination). *Scale bar*, 50 μm. Quantitative analysis of *Kiss1r* mRNA in GnRH neurons demonstrated the percentage of double-labeled cells (B) and the relative expression of *Kiss1r* mRNA in GnRH neurons (C, reflected by the number of *silver grains* per GnRH neuron) was similar in luteal and late-follicular phase ewes (n = 4–5 per group). Data are the ± SEM.

LH and GnRH responses to kisspeptin in luteal and late-follicular phase ewes

We aimed to determine whether the LH surge is characterized by a shift in the GnRH response to kisspeptin. Kisspeptin treatment increased the concentrations of GnRH and LH in all animals compared with the vehicle-treated controls (Fig. 3, A and B). The LH response to kisspeptin treatment differed significantly with the reproductive phase ($P < 0.05$). Thus, the LH response to kisspeptin treatment was greater during the late-follicular phase compared with the luteal phase. AUC analysis confirmed the LH response to kisspeptin was greater in late-follicular (3.0-fold higher) phase ewes compared with luteal phase ewes ($P < 0.05$, Fig. 3C). The GnRH response differed with treatment only ($P < 0.001$), and this was also reflected in the AUC data, in which the response to kisspeptin treatment was similar in luteal and late-follicular phase ewes.

Kiss1r expression on GnRH neurons in luteal and late-follicular phase ewes

To assess whether variability in GnRH/LH response to kisspeptin reflects a change in kisspeptin sensitivity, we examined the expression of *Kiss1r* mRNA on GnRH neurons with double-label *in situ* hybridization. GnRH positive cell bodies were located in their typical distribution within the forebrain-POA. The mean number of identifiable *GnRH* mRNA expressing neurons was similar between groups (luteal, 30 ± 5 cells; late follicular, 31 ± 8 cells). These same cells also showed clusters of silver grains reflecting *Kiss1r* mRNA (Fig 4A). Quantitative analysis showed that 78 ± 2% of all iden-

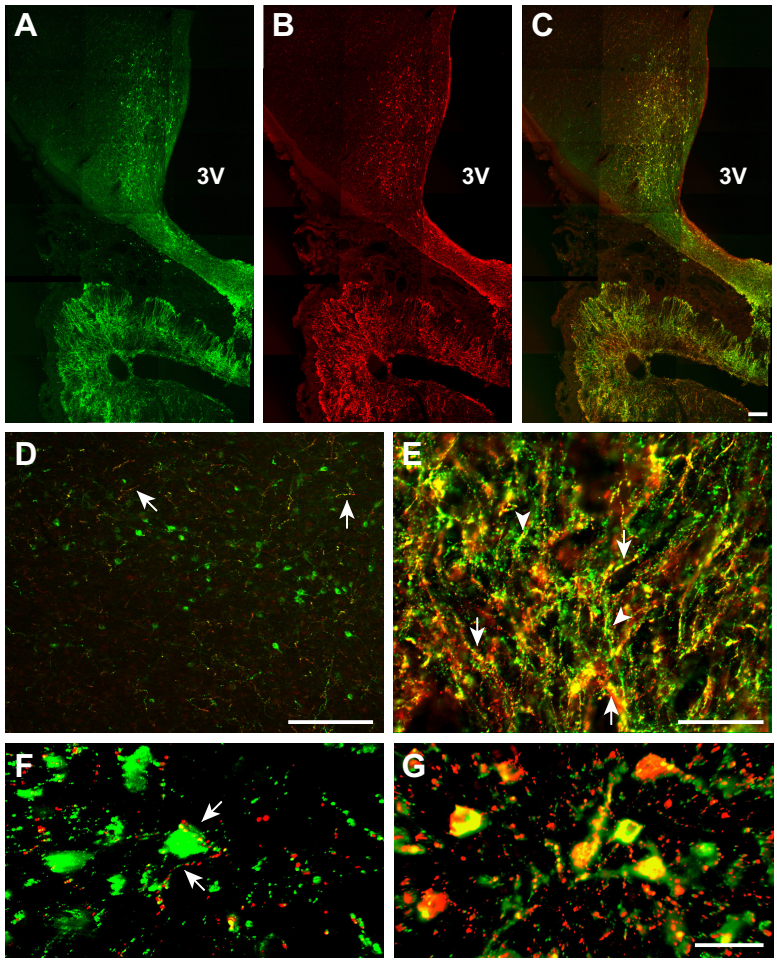

FIG. 5. Coexpression of kisspeptin and NKB indicate kisspeptin fibers in the external zone of the ME originate from the ARC. A–C, Dual immunofluoresence, low-power composite images showing kisspeptin (*green*, A) and NKB (*red*, B) coexpression (*yellow* merge, C) in cells of the ARC as well as fibers in the ME. D, Merged image showing no kisspeptin/NKB colocalization in the POA. *Arrows* indicate the presence of kisspeptin/NKB fibers. E, Merged high-power image showing kisspeptin/NKB fibers in the external zone of the ME. *Arrows* indicate clear examples of kisspeptin/NKB colabeled fibers (*yellow*). *Arrowheads* indicate kisspeptin single labeled fibers (*green*). F, Z-stack apotome image showing a kisspeptin cell (*green*) in the POA with a kisspeptin/NKB terminal in close apposition. G, Z-stack apotome image showing kisspeptin/NKB cells and fibers (*yellow*) in the ARC. *Scale bars* (A-D), 200 μm; (E), 100 μm; (F and G), 20 μm. 3V, Third ventricle.

tifiable *GnRH* mRNA-expressing neurons counted also expressed *Kiss1r* mRNA in luteal phase ewes (Fig. 4B). The percentage of *GnRH*/*Kiss1r* was similar in late-follicular phase ewes (86 ± 2%; Fig. 4B). The relative expression of *Kiss1r* mRNA (number of silver grains) on GnRH neurons was also similar in luteal and late-follicular phase ewes (Fig. 4C).

Anatomical origin of kisspeptin fibers in the ME

To examine the origin of kisspeptin fibers in the ME, we used dual-label immunohistochemistry and anterograde tracer injections. Virtually all kisspeptin-immunoreative cells in the ARC were colocalized with NKB (99 ± 1%, mean cells counted per animal 104 ± 18; Fig. 5, A–C), whereas no kisspeptin/NKB colocalization was apparent in the POA (mean cells counted per animal 74 ± 21; Fig. 5D). Examination of the ME revealed a high abundance of kisspeptin-immunoreactive fibers with many colabeled with NKB (Fig. 5E), indicating an ARC origin. Quantitative assessment showed 30 ± 4% overlap of kisspeptin staining with NKB in the ME. Kisspeptin/NKB-immunoreactive fibers were also present in the dorsal medial hypothalamus, ventromedial hypothalamus, and bed nucleus of the stria terminalis (data not shown) as well as the ARC and scattered expression was seen in the POA (Fig. 5D). In the POA, quantitative assessment showed 1.4 ± 0.4% overlap of kisspeptin staining with NKB. Close apposition of kisspeptin/NKB fibers was noted on 10 ± 4% of kisspeptin cell bodies (mean cells counted per animal 74 ± 21; Fig. 5B). In the ARC, examples of kisspeptin/NKB fiber apposition with kisspeptin/NKB cells were also noted (Fig. 5G). Injection of the anterograde neurotracer BDA into the ARC confirmed that kisspeptin cells of this nucleus project to the ME (Fig. 6). Figure 6A shows BDA injection sites located in the ARC in the region of kisspeptin cells (Fig. 6B) and anterograde labeled fibers in the ME (Fig. 6B). Kisspeptin and BDA colocalized fibers were identified in the ME of all animals (Fig. 6, C and D).

Kiss1r expression on *Kiss1* neurons

To assess whether kisspeptin fiber appositions onto kisspeptin cell bodies reflect a functional unit, we examined the expression of *Kiss1r* mRNA on kisspeptin (*Kiss1* mRNA) neurons with double-label *in situ* hybridization. *Kiss1* mRNA-positive cell bodies were located in the POA and ARC, but virtually none contained clusters of silver grains reflecting *Kiss1R* mRNA (mean cells counted per animal, POA 101 ± 44, ARC 160 ± 62; Fig. 7A). Quantitative analysis of *Kiss1* mRNA-expressing neurons showed that 0.9 ± 0.4% in the POA and 0.9 ± 0.9% in the ARC also expressed *Kiss1r* mRNA.

GnRH response to kisspeptin stimulation in cultured ME explants

To determine whether kisspeptin fibers in the ME may stimulate GnRH secretion, we sought to examine the effect of kisspeptin on the isolated ME in culture. Incubation of ME with kisspeptin for 1 h resulted in a significant increase in GnRH release (Fig. 8A). Specifically we show

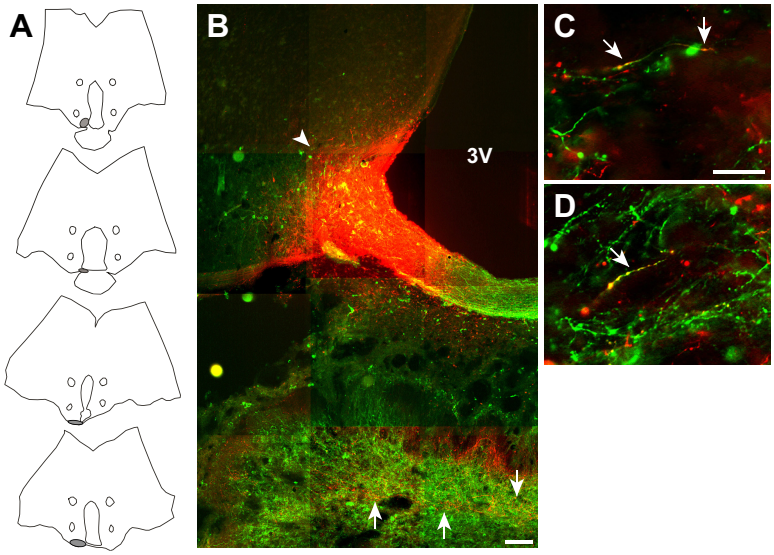

FIG. 6. BDA injections into the ARC demonstrate kisspeptin fibers in the external zone of the ME. A, Schematic representation of injection sites from four animals. *Gray circles* indicate injection site in the ARC. B, Low-power composite images showing kisspeptin (*green*, A) and BDA (*red*, B) immunofluoresence. *Arrow* indicates the site of BDA injection overlapping kisspeptin neurons. *Arrowheads* indicate the presence of kisspeptin and BDA-immunoreactive fibers in the external zone of the ME. C and D, Merged high-power images showing kisspeptin/BDA fibers in the external zone of the ME. *Arrows* indicate clear examples of kisspeptin/BDA colabeled fibers (*yellow*). *Scale bars* (B), 200 μm; (C and D), 100 μm. 3V, Third ventricle.

the lowest effective dose of kisspeptin was 100 nM, resulting in a greater than 3-fold increase in the GnRH concentration in culture media. Similar results were also achieved with 1000 nM kisspeptin. Close association between kisspeptin and GnRH-immunoreactive fibers were readily detectable in the ME (Fig. 8C) as previously described (2, 19).

Discussion

It is clear kisspeptin signaling is fundamental to the reproductive system (9, 10). Specifically kisspeptin cells in the hypothalamus are strong candidates to act as key conduits, providing the missing link in the steroid feedback control of GnRH secretion (1, 2). Concerning estrogen-positive feedback, which drives the preovulatory GnRH/LH surge, the critical role of kisspeptin signaling in this phenomenon has recently been subjected to debate (as indicated in the introductory text). To test the specific mandatory role for kisspeptin in estrogen positive feedback and the LH surge, we administered kisspeptin antagonist to ewes subjected to an EB-induced LH surge. Our results show kisspeptin antagonist treatment reduces the magnitude of the LH surge, indicating an important (but not requisite) role for kisspeptin in the underlying E2-positive feedback mechanism. This is consistent with the observation that other neuronal systems also play a role in the positive feedback effect of E2 on GnRH secretion. Thus, blockade of α-1-noradrenergic action also reduces the magnitude of the LH surge (33). Our data are also consistent with a previous report in OVX rats in which infusion of antirat kisspeptin monoclonal antibody significantly reduced (but did not completely prevent) the E2-induced LH surge (34). In an earlier report, the same treatment completely prevented the surge (35); however, this experiment was performed in intact rats, and again the determination of a specific role of kisspeptin signaling on the positive feedback mechanism was not possible.

One possible explanation for the incomplete effect of kisspeptin antagonist treatment on the LH surge could be that the infusion dose does not fully saturate all *Kiss1r*, leading to partial blockade of kisspeptin signaling. This seems unlikely because the high doses of antagonist used in this study were successful in completely blocking the pulsatile secretion of LH in OVX ewes. Alternatively, the rise in kisspeptin signaling during the surge is likely to be much greater than that during pulsatile LH secretion, so a greater dose of kisspeptin antagonist may be required. Another possibility is that our experimental time frame was not optimal for complete blockade of kisspeptin signaling at the most optimal point to prevent the surge. Based on Fos labeling of cells in the A1 nucleus of the brainstem and ARC, it is clear that our EB injections activate cells within 1 h (36). It seems most likely, however, that the positive feedback event involves more than one neuronal system, although it is clear that the kisspeptin cells of the ARC and POA play a significant role, at least in this species (6, 21, 37) and the nonhuman primate (8).

From our data we can conclude that kisspeptin antagonist-treated ewes retain some ability to exhibit the positive-feedback effects of E2 and mount an LH surge but experience an inhibition in postovariectomy LH pulse secretion. This suggests kisspeptin signaling is required for the tonic/pulsatile release of GnRH and LH. Kisspeptin cells in the ARC are well placed to drive the estrogen-negative feedback signals that control the tonic pulsatile release of GnRH (1, 2). This has been shown through genetic deletions of *Kiss1r* in mice (12) and appears to be true for multiple species by use of kisspeptin antagonists (13, 38). Importantly, the present data do not directly test whether *Kiss1* neurons in the ARC mediate the negative-feedback actions of estrogen but are consistent with this hypothesis. This is substantiated through evidence of *Kiss1* expression down-regulation in the ARC by E2 in

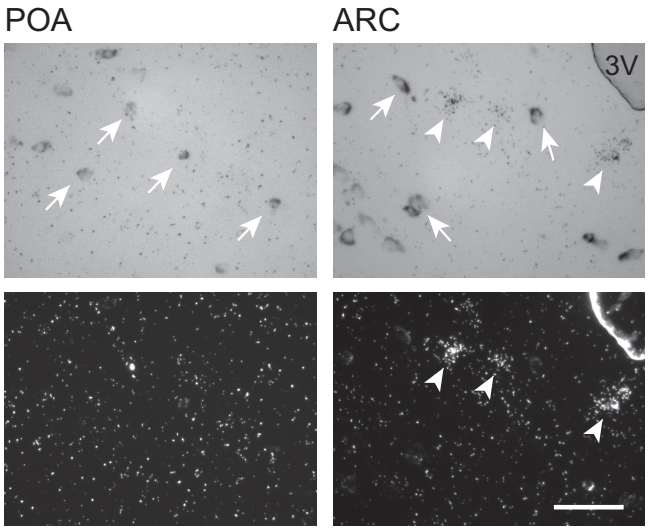

FIG. 7. *Kiss1r* mRNA is not coexpressed in *Kiss1* neurons. Representative bright-field photomicrographs show cells expressing *Kiss1* mRNA in the POA and ARC indicated by *arrows* (*top panels*, visualized with nitroblue tetrazolium substrate) and corresponding dark-field photomicrographs (*lower panels*) show expression of *Kiss1r* mRNA (clusters of *silver grains*). *Arrowheads* indicate *Kiss1r*-positive cells not expressing *Kiss1* (note: *silver grains* can also be seen in bright field illumination). *Scale bar*, 50 μm. 3V, Third ventricle. Quantitative analysis of *Kiss1* neurons demonstrated less than 1% (POA, 0.9 ± 0.4%; ARC, 0.9 ± 0.9%) coexpressed *Kiss1r* mRNA (n = 4).

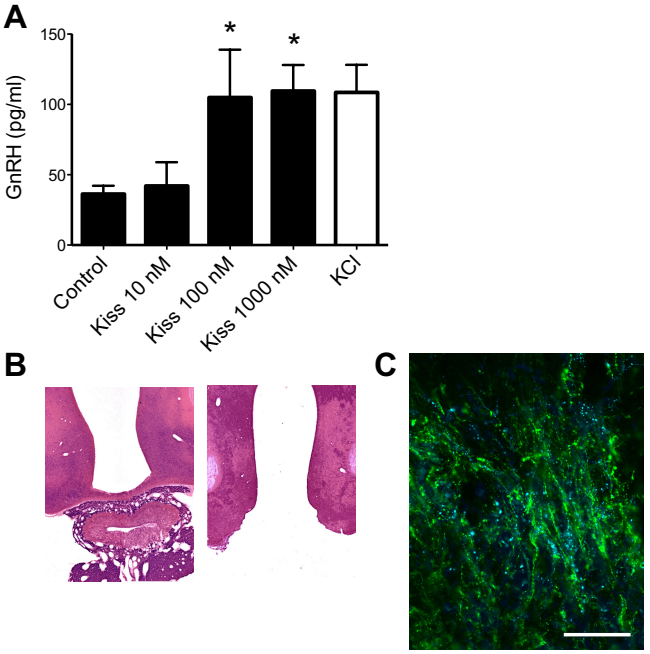

FIG. 8. Kisspeptin stimulates GnRH release in the isolated ME. A, Direct stimulatory effect of kisspeptin on GnRH release from ME explants. ME was challenged with different concentrations of kisspeptin (10, 100, 1000 nM) or control (DMEM alone, n = 5–7 per group). All MEs were subsequently exposed to KCl (60 nM). Data are the mean ± SEM. *, $P < 0.05$. B, One ME-intact hypothalamus and one hypothalamus after ME dissection stained with hematoxylin and eosin detailing the ME dissection. C, Dual-immunofluoresence image showing kisspeptin- (*green*) and GnRH (*cyan*)-expressing fibers in close apposition in the external zone of the ME. *Scale bar*, 100 μm.

mice (39, 40) and sheep (5, 26) and virtually all these cells possessing estrogen receptor-α (3, 39, 40). Moreover, growing evidence suggests that ARC kisspeptin cells drive pulsatile GnRH secretion, and the neuropeptides NKB and dynorphin, which are coexpressed on ARC *Kiss1* cells (24), act autosynaptically on these neurons to synchronize the release of kisspeptin (41–43).

We show that kisspeptin can directly stimulate GnRH release from the isolated ovine ME in an *ex vivo* culture model. Similar results have previously been achieved using mouse cultured mediobasal hypothalami (20). Using the isolated ME, we greatly reduce the chance of cellular components acting on GnRH fibers, and such data are important because they offer novel pathways from kisspeptin cells to GnRH neurons. The precise anatomical origins of kisspeptin inputs to GnRH neurons are yet to be fully understood, but it is clear that there is input to perikarya. Kisspeptin fibers make connections and close appositions to GnRH neurons in mice (44), rats (35), sheep (26), and monkeys (19). Importantly, direct neuronal inputs to GnRH cell bodies in the ventral medial POA (in which the majority of GnRH neurons are found) do not appear to arise from key cells located in the ARC in mice (18) or sheep (25). In sheep, POA kisspeptin cells provide substantial input to GnRH cells in the ventromedial POA, whereas ARC kisspeptin cells do not (17). It is plausible then that ARC kisspeptin neurons find their way to GnRH neurons through terminal-to-terminal communication at the ME and regulate GnRH secretion by volume control. We support this hypothesis by showing kisspeptin fibers present in the external zone of the ME, in which they lie in close apposition to GnRH neuronal fibers (2, 19). These projections appear to originate substantially from the ARC, based on dual localization with NKB. Interestingly, quantitative assessment of kisspeptin/NKB fibers in the ME indicated only 30% overlap. We believe this technique may underestimate the colocalization of fibers because immunostaining is rarely continuous along the entire fiber length. Similarly, data have recently been generated in rats, indicating kisspeptin/NKB fiber colocalization in the internal zone of the ME (7). Alternative indirect pathways may also exist because we show a small number of ARC kisspeptin fibers appose kisspeptin cells in the POA. Thus, at least a subset of kisspeptin cells in the POA may form an interneuronal bridge linking ARC kisspeptin cells to GnRH cell bodies. This level of communication, however, appears unlikely because we show virtually no *Kiss1r* expression on kisspeptin cells in the POA. Consistent with this, in mouse models, anteroventral paraventricular nucleus neurons (including kisspeptin neurons) do not respond to kisspeptin (45). Similarly, virtually no *Kiss1r* expression was seen on kisspeptin cells in the ARC, indi-

cating the autosynaptic action proposed for NKB and dynorphin on these neurons to synchronize the release of kisspeptin is unlikely to include an autosynaptic action of kisspeptin. Interestingly, recent data show kisspeptin directly excites anorexigenic proopiomelanocortin neurons but inhibits orexigenic neuropeptide Y neurons (46), and we have shown reciprocal connections between these and kisspeptin neurons (28). Whether proopiomelanocortin or neuropeptide Y neurons possess *Kiss1r* is yet to be determined.

In humans and sheep, the LH response to kisspeptin treatment appears to be highest during the preovulatory phase of the menstrual/estrous cycle (15, 16), but it was unclear as to whether this was due to increased GnRH secretion. We show the GnRH secretory response (measured directly in portal blood) to kisspeptin was similar in the luteal and late-follicular phases of the estrous cycle of the ewe. Consistent with this, *Kiss1r* mRNA expression on GnRH neurons was similar in luteal and late-follicular phase animals. GnRH has its greatest effect (stimulating LH secretion) during the late-preovulatory phase of cycle in humans (47) and sheep (48), and this is substantially due to increased pituitary sensitivity to GnRH just before the onset of the LH surge. Alternatively, it may be possible that kisspeptin can directly stimulate LH secretion at the pituitary around the time of the LH surge. We feel this is unlikely because kisspeptin failed to stimulate LH secretion in a follicular-like phase hypothalamo-pituitary-disconnected ewe (49). Overall, the present data agree well with the observation that the increased response to kisspeptin in terms of LH secretion is due to sensitization of the pituitary gonadotrope to GnRH in the late-follicular phase.

Since 2003, kisspeptins have been cast to the forefront of neuroendocrine research, and remarkable advances have been made in our understanding of the reproductive axis. We show herein that kisspeptin signaling is a fundamental prerequisite for generation of the full preovulatory LH surge but may not be the only neuronal factor involved. The sensitivity of kisspeptin effects on GnRH secretion into the portal blood was unchanged between the luteal to the late-follicular phase of the estrous cycle (just before the LH surge), highlighting the change in sensitivity of the gonadotropes to GnRH at this time. Kisspeptin fibers are present in the external zone of the ME in close association with GnRH fibers, and we further show evidence that a significant proportion of kisspeptin fibers in the ME originate from the ARC. Importantly, we show direct effects of kisspeptin on GnRH secretion from isolated ME, indicating GnRH release from neuronal fibers. These data provide a novel model by which ARC kisspeptin cells may directly stimulate GnRH secretion.

Acknowledgments

We thank Bruce Doughton, Lynda Morrish, Elaine Chase, Alexandra Rao, and Alda Pereira for their technical assistance.

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This work was supported by the National Health and Medical Research Council of Australia Project Grant 606538. J.T.S. is supported by an Australian Research Council Future Fellowship FT0990986.

Disclosure Summary: The authors have nothing to disclose.

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