

## Evidence That Dopamine Acts via Kisspeptin to Hold GnRH Pulse Frequency in Check in Anestrous Ewes

Robert L. Goodman, Matthew J. Maltby, Robert P. Millar, Stanley M. Hileman, Casey C Nestor, Brant Whited, Ashlie S. Tseng, Lique M. Coolen, and Michael N. Lehman

Departments of Physiology and Pharmacology (R.L.G., S.M.H., C.C.N., B.W., A.S.T.), Robert C. Byrd Health Sciences Center, Morgantown, West Virginia 26506; Department of Anatomy and Cell Biology (M.J.M.), Schulich School of Medicine and Dentistry, The University of Western Ontario, London, Ontario, Canada N6A 5C1; Mammal Research Institute (R.P.M.), University of Pretoria, Pretoria 0002, South Africa; University of Capetown/Medical Research Council Receptor Biology Unit (R.P.M.), University of Cape Town, 7701 Cape Town, South Africa; Centre for Integrative Physiology (R.P.M.), University of Edinburgh, Edinburgh EH16 4SB, Scotland, United Kingdom; and Department of Molecular and Integrative Physiology (L.M.C., M.N.L.), University of Michigan, Ann Arbor, Michigan 48109

Recent work has implicated stimulatory kisspeptin neurons in the arcuate nucleus (ARC) as important for seasonal changes in reproductive function in sheep, but earlier studies support a role for inhibitory A15 dopaminergic (DA) neurons in the suppression of GnRH (and LH) pulse frequency in the nonbreeding (anestrous) season. Because A15 neurons project to the ARC, we performed three experiments to test the hypothesis that A15 neurons act via ARC kisspeptin neurons to inhibit LH in anestrus: 1) we used dual immunocytochemistry to determine whether these ARC neurons contain D2 dopamine receptor (D2-R), the receptor responsible for inhibition of LH in anestrus; 2) we tested the ability of local administration of sulpiride, a D2-R antagonist, into the ARC to increase LH secretion in anestrus; and 3) we determined whether an antagonist to the kisspeptin receptor could block the increase in LH secretion induced by sulpiride in anestrus. In experiment 1, 40% of this ARC neuronal subpopulation contained D2-R in breeding season ewes, but this increased to approximately 80% in anestrus. In experiment 2, local microinjection of the two highest doses (10 and 50 nmol) of sulpiride into the ARC significantly increased LH pulse frequency to levels 3 times that seen with vehicle injections. Finally, intracerebroventricular infusion of a kisspeptin receptor antagonist completely blocked the increase in LH pulse frequency induced by systemic administration of sulpiride to anestrous ewes. These results support the hypothesis that DA acts to inhibit GnRH (and LH) secretion in anestrus by suppressing the activity of ARC kisspeptin neurons. (*Endocrinology* 153: 5918–5927, 2012)

**R**eproductive function in many mammals occurs seasonally so that the young are born into an environment favorable for their survival. Most mammals living at some distance from the equator use photoperiodic cues to synchronize periods of fertility to the appropriate season (1, 2). This photoperiodic information is transduced by the pineal gland into an endocrine signal: the daily pattern of melatonin secretion. Because melatonin is secreted only at

night, the duration of the elevated melatonin concentrations is used as an index of external photoperiod (1, 2). Early work indicated that the sites of action of melatonin to control reproduction are species specific (2–5), but more recent work has focused on possible actions in the pars tuberalis (6, 7). However, the interneurons connecting these melatonin-responsive systems to the GnRH neurons whose secretion is altered by photoperiod remain unclear.

ISSN Print 0013-7227 ISSN Online 1945-7170

Printed in U.S.A.

Copyright © 2012 by The Endocrine Society

doi: 10.1210/en.2012-1611 Received June 7, 2012. Accepted September 12, 2012.

First Published Online October 4, 2012

Abbreviations: AHA, Anterior hypothalamic area; ARC, arcuate nucleus; AVPV, anteroventral periventricular nucleus; DA, dopaminergic; D2-R, dopamine receptor; E<sub>2</sub>, estradiol; ICC, immunocytochemistry; icv, intracerebroventricular; ir, immunoreactive; Kiss1r, kisspeptin 1 receptor; KNDy, kisspeptin/neurokinin B/dynorphin; MBH, mediobasal hypothalamus; NGS, normal goat serum; OVX, ovariectomy; POA, preoptic area; SD, short-day photoperiod; SON, supraoptic nucleus of the hypothalamus.

Recent work on potential mediators of the photoperiodic control of GnRH secretion has focused on kisspeptin neurons (7, 8). Since kisspeptin was discovered as a critical stimulator of GnRH secretion in humans (9, 10), the physiological role of kisspeptin neurons has been extensively studied in mice and rats (11). In these nonphotoperiodic rodents, two populations of kisspeptin neurons are thought to mediate different actions of gonadal steroids: kisspeptin neurons in the arcuate nucleus (ARC) mediate steroid negative feedback, whereas the more rostral populations [in the anteroventral periventricular nucleus (AVPV) and adjacent areas] are critical for the positive feedback actions of estradiol ( $E_2$ ) that induce the preovulatory GnRH and LH surges (11–13).

The population of ARC kisspeptin neurons has also been implicated in seasonal breeding in female sheep. In ewes, seasonal changes in GnRH secretion reflect marked seasonal alterations in response to  $E_2$ -negative feedback (1, 2), with  $E_2$  strongly inhibiting GnRH pulse frequency in the anestrus (nonbreeding) season but not during the breeding season (14, 15). The number of kisspeptin-containing neurons in the ARC, but not the preoptic area (POA), of  $E_2$ -treated ovariectomized (OVX+E) ewes is decreased in anestrus (16, 17), due largely to an increased inhibition by  $E_2$  in anestrus (16). These data, together with the observation that exogenous kisspeptin induced ovulation in ovary-intact anestrus ewes (18, 19), support an important role for this kisspeptin population in seasonal breeding in the ewe.

Although recent work has focused on the role of kisspeptin neurons, a considerable body of evidence also supports a role for the A15 dopaminergic neurons located in the retrochiasmatic area in seasonal breeding in ewes (15). Briefly, these neurons are stimulated by  $E_2$  in anestrus, but not the breeding season (20), and mediate  $E_2$ -negative feedback in anestrus ewes (21, 22) via D2 dopamine receptors (D2-Rs) (23, 24). Because A15 neurons do not contain estrogen receptors (25, 26), a number of studies have focused on estrogen-responsive afferents and have identified these in the ventromedial POA (15, 27) and retrochiasmatic area (15, 28, 29). The efferents of the A15 have received less study, but their major projections are posterior to the median eminence (15, 30) and ARC (15). In light of the A15 projections to the ARC and the seasonal changes in kisspeptin expression in this nucleus, we hypothesize that A15 dopaminergic neurons inhibit GnRH pulse frequency via the ARC kisspeptin populations.

This study included three experiments to test different aspects of this hypothesis. First, we used dual immunocytochemistry (ICC) to determine whether ARC kisspeptin neurons contain D2-R; D2-R expression in GnRH neurons was also of interest because of possible monosynaptic

connections between the A15 and these neurons (15). One practical paradox in assessing D2-R expression in kisspeptin neurons is that kisspeptin expression itself is suppressed in anestrus ewes (16, 17) and, if this inhibition occurs via A15 dopaminergic (DA) neurons, it would be particularly effective in any kisspeptin neurons that contain D2-R. To overcome this problem, we took advantage of the fact that these ARC kisspeptin neurons also contain neurokinin B and dynorphin (31) and thus are called KNDy (kisspeptin/neurokinin B/dynorphin) neurons (32). Because 95% of kisspeptin neurons contain dynorphin and vice versa (31), and dynorphin does not play a role in seasonal changes in  $E_2$ -negative feedback (15), we used it as a marker for KNDy neurons in this experiment. Second, we tested the hypothesis that DA acts in the ARC to hold LH pulse frequency in check in anestrus ewes using local administration of a D2-R antagonist into this area. Finally, we tested the role of kisspeptin in mediating dopaminergic effects by determining whether a kisspeptin receptor antagonist would inhibit the stimulatory actions of a D2-R antagonist on LH pulse frequency.

## Materials and Methods

### Animals

Adult mixed-breed blackface ewes were maintained in an open barn with access to water and fed silage daily. They were moved indoors 3–7 d before surgeries and remained there for the duration of each experiment. Indoors, ewes were fed alfalfa pellets to maintain weight and given water and supplemental minerals *ad libitum*. Lights were adjusted every 2 wk to mimic the duration of natural lighting. For experiment 1, tissue was collected from anestrus (May) and breeding season (November) ewes. Experiments 2 and 3 were performed from the beginning of June through early July in anestrus ewes. The seasonal status of each ewe was determined based on estrous behavior and/or status of the ovaries at the time of ovariectomy (OVX) or tissue collection. Blood samples (4–5 ml) were collected by jugular venipuncture (experiment 2) or jugular catheter (experiment 3) into heparinized tubes, and plasma was collected and stored at  $-20\text{ C}$  until assayed. All procedures were approved by the West Virginia University Animal Care and Use Committee and followed National Institutes of Health guidelines for the use of animals in research.

### Surgeries

All surgical procedures were performed with sterile techniques using 1–4% isoflurane as anesthesia. Ovaries were removed via midventral laparotomy as previously described (20). Stereotaxic neurosurgical procedures (27) were used for chronic implantation of bilateral 18-gauge guide tubes just above the ARC (experiment 2) or a 16-gauge needle into one lateral ventricle (experiment 3). For guide tube implantations, the surface of the skull was exposed and a hole (1 cm in diameter) drilled and punched in the bone just rostral to bregma to expose the surface

of the brain. The sagittal sinus was ligated; a cannula inserted into a lateral ventricle; and radioopaque dye, Omnipaque 350 (Iohexol, Winthrop, NY) was injected into it. The bilateral guide tubes were lowered into position (2.0 mm from midline, 2.0 mm dorsal to the floor of the third ventricle, just above the start of the infundibular recess) using lateral and frontal x-ray radiography. For implantation of one lateral ventricle, a smaller hole (0.3 cm diameter) was drilled 5 mm rostral and 4 mm lateral to bregma and the exposed dura cauterized. A 1.25-in. long 16-gauge needle, with a water-filled reservoir, was then lowered until liquid flowed into the ventricle. Radioopaque dye was injected and the dorsal-ventral position of the needle tip adjusted based on a lateral x-ray. ARC guide tubes and lateral ventricle cannulae were cemented in place using dental acrylic and stainless steel screws, protected with a plastic cap, and occluded. The animals were treated with dexamethasone, penicillin, and banamine from 1 d before the surgery to 3 d afterward, as previously described (27).

### Tissue collection

To collect tissue for histological and ICC analyses, ewes were heparinized (25,000 U iv 10 min apart) and killed with an iv overdose of pentobarbital (~7 g). When they stopped breathing, their heads were removed and immediately perfused via internal carotids with 6 liters of 4% paraformaldehyde in 0.1 M phosphate buffer containing 0.1% NaNO<sub>3</sub>. Tissue blocks were stored overnight in fixative at 4 C and then in 30% sucrose at 4 C. After they had been infiltrated with sucrose, 45- $\mu$ m-thick frozen coronal sections were cut using a freezing microtome. For histological identification of microinjections (experiment 2) and lateral ventricle infusions (experiment 3), every fifth section was mounted on slides, cleared, and stained with cresyl violet. For ICC, six series of sections (270  $\mu$ m apart) were stored at -20 C in cryoprotectant until processed.

### Experiments

#### **Experiment 1. Do kisspeptin and/or GnRH neurons contain D2-R, and, if so, does the expression change with season?**

Anestrous and breeding season ewes (n = 5/group) were OVX and a 3-cm-long SILASTIC brand capsule (Dow Corning Corp., Midland MI) containing E<sub>2</sub> (16, 20) inserted sc at the end of surgery. As in previous work (1, 5, 16, 20), OVX+E ewes were used for this seasonal comparison to eliminate the possible confounding effects of seasonal variations in endogenous steroid concentrations. Three weeks later, these OVX+E ewes were killed and paraformaldehyde-fixed tissue collected for dual immunocytochemistry. As noted above, we used dynorphin as a marker for ARC kisspeptin-containing neurons to avoid the confounding effects of seasonal differences in kisspeptin expression in OVX+E ewes (16, 31). The GnRH and dynorphin antibodies used for dual ICC have previously been validated for use in sheep (33, 34), but the antibody against D2-R has not. No antigen was available for a preabsorption control, so we first performed Western blot analysis with this antibody on protein extracted from neural tissue of an ovary-intact anestrous ewe to determine whether it cross-reacted with any other proteins in this species. A sample of the ARC was obtained from fresh frozen tissue using a micropunch technique, homogenized, and the protein ex-

tracted. This protein, and protein from a whole-cell lysate of human neuroblastoma cells (sc-2410; Santa Cruz Biotechnology, Santa Cruz, CA) as a positive control, were subjected to Western analysis (see Supplemental Methods, published on The Endocrine Society's Journals Online web site at <http://endo.endojournals.org>) using a mouse anti-D2-R (Santa Cruz Biotechnology; sc-5303) at the final dilutions of 1:500, 1:1000, or 1:2000.

Immunocytochemistry was performed using free-floating tissue sections washed of cryoprotectant in 0.1 M PBS for 3 h at 15-min intervals. Tissue sections were then incubated in 1% H<sub>2</sub>O<sub>2</sub> in 0.1 M PBS for 10 min to block endogenous peroxidase activity and rinsed in 0.1 M PBS four times for 5 min each. Sections were then rinsed for 1 h in normal goat serum (NGS) (0.1 M PBS with 4% Triton X-100 and 4% normal goat serum) and subsequently incubated with mouse anti-D2-R antibody (Santa Cruz Biotechnology; sc-5303) at 1:100 in NGS for 17 h. Tissue sections were then rinsed (0.1 M PBS for 5 min, four times) and rinsed again after each subsequent step. Next, the sections were incubated in the secondary antibody, biotinylated goat anti-mouse (Vector Laboratories, Burlingame, CA), at 1:500 in NGS for 1 h. To amplify the signal, sections were first incubated in ABC-elite (Vector Laboratories) 1:500 in PBS for 1 h. Next, the signal was amplified using the tyramide signal amplification system by incubating sections with biotinylated tyramine (PerkinElmer, Waltham MA) at 1:250 in PBS containing 0.003% H<sub>2</sub>O<sub>2</sub> for 10 min. Tissue sections were then incubated for 30 min in Alexa 488 Fluor-conjugated streptavidin (Molecular Probes, Eugene OR) at 1:100 in PBS.

Tissue sections were next incubated with either 1:500 mouse anti-GnRH (Sternberger Monoclonals, Inc., Lutherville MD; catalog no. SMI-41R, lot no. 3) or 1:500 rabbit antidynorphin A (Phoenix Pharmaceuticals, Inc., Phoenix, AZ; H-021-03, lot no. 00355) antiserum in NGS for 17 h. After being washed in PBS, the sections were incubated for 30 min in the secondary antibody Alexa Fluor 555 goat antimouse or antirabbit, respectively (Molecular Probes) at 1:100 in PBS (with 4% Triton X-100). After processing, the tissue sections were mounted on glass slides using 0.3% gelatin, coverslipped using gelvatol, and stored in the dark at 4 C. Controls for immunocytochemistry included omission of each primary antibody from this protocol, and the Western blot described above was used to confirm the specificity of the D2-R antibody.

#### **Experiment 2. Does DA act in the ARC to hold LH pulse frequency in check in anestrous ewes?**

Chronic bilateral guide tubes were implanted just above the ARC in ovary-intact anestrous ewes (n = 7) in early June. Starting approximately 2 wk later, the effects of bilateral microinjections of the D2-R antagonist, sulpiride, into the ARC were determined. Four doses of sulpiride were tested (0, 2, 10, and 50 nmol/side) in each ewe, with the treatment order randomized and 3–4 d between each treatment. Sulpiride (Sigma-Aldrich Chemical Co., St. Louis, MO) was dissolved in 0.1 M tartaric acid at a concentration of 50 nmol per 300 nl and stored at 4 C. On the day before treatments, the stock solution was diluted to 10 nmol per 300 nl and 2 nmol per 300 nl with 0.1 M tartaric acid, so all doses of sulpiride were given in the same vehicle. Blood samples were collected every 12 min for 36 min before and 4 h after the rapid (less than 1 min) injection of 300 nl of vehicle or sulpiride

to both sides of the ARC. At the end of the experiment, ewes were killed and tissue collected for histological determination of injection sites.

### Experiment 3. Does kisspeptin mediate the stimulatory actions of a D2-R antagonist in anestrus ewes?

To test this hypothesis, we administered a kisspeptin 1 receptor (Kiss1r) antagonist (p-271), which is identical to a previously described antagonist (p-234) (35) but which has a penetratin peptide sequence attached to the N terminus to facilitate transfer across the blood-brain barrier (36); both peptides inhibited LH secretion in ewes (35, 37). A stock solution of antagonist (6.66 mg/ml in sterile saline) was stored at  $-20^{\circ}\text{C}$  and diluted to working concentrations (in sterile saline) the morning of its use. Because no data on the effects of p-271 in sheep were available when this work was done, we first determined an effective dose in OVX ewes.

#### Experiment 3a. What infusion rate of the Kiss1r antagonist inhibits LH secretion in OVX ewes?

A 16-gauge needle was chronically implanted into the right lateral ventricle of six OVX ewes in early anestrus (April). Two weeks later, the jugular vein of each ewe was catheterized, and the next day blood samples collected every 12 min for 10 h. Sterile saline (120  $\mu\text{l/h}$ ) was infused into the lateral ventricle for 0–2 h in all six ewes, followed by p-271 (20, 40, or 60  $\mu\text{g/h}$ ,  $n = 2/\text{treatment}$ ) for 2–5 h during the blood collections. Infusions were done with battery-operated pumps strapped to each ewe's back through sterilized line and 22 stainless steel tubing that extended to the tip of the needle in the lateral ventricle (33). At the end of the experiment, fixed tissue was collected and the location of each needle in the lateral ventricle confirmed histologically.

#### Experiment 3b. Does kisspeptin mediate stimulatory actions of a D2-R antagonist in anestrus ewes?

A chronic 16-gauge needle was implanted into the right lateral ventricle of six ovary-intact anestrus ewes in early June. Approximately 2 weeks later, sulphiride [12 mg/ml (35  $\mu\text{mol/ml}$ ) in 0.1 M tartaric acid] and Kiss1r antagonist (0.5 mg/ml in sterile saline) solutions were prepared and the jugular vein of each ewe was catheterized. The next day, blood samples were collected every 12 min for 6 h; sterile saline (120  $\mu\text{l/h}$ ,  $n = 3$ ) or Kiss1r antagonist (60  $\mu\text{g/h}$ ,  $n = 3$ ) was infused from h 2–6, and at h 3, all six ewes were given an im injection of sulphiride [1.2 mg/kg (3.5  $\mu\text{mol/kg}$ )]; this dose reliably increases LH pulse frequency in ovary-intact ewes (27). At the end of blood collection, the infusion lines and catheter were removed, and the sampling and treatment procedures were then replicated 6 d later using a cross-over design for Kiss1r antagonist treatment so that ewes receiving vehicle in the first replicate were given antagonist in the second, and vice versa. At the end of the experiment, fixed tissue was collected and location of needles in the lateral ventricle confirmed histologically.

### Analytical procedures

#### Assays

LH and prolactin concentrations were measured using reagents provided by the National Hormone and Peptide Program

(Torrance, CA) as previously described (38, 39). LH was measured in duplicate aliquots (50–200  $\mu\text{l}$ ) of all samples collected in experiments 2 and 3; assay sensitivity averaged 0.07 ng/tube (NIH S24), and intra- and interassay coefficients of variation were 5.3 and 13.8%, respectively. Prolactin concentrations were measured in two assays using duplicate 5- $\mu\text{l}$  aliquots of every other sample collected during experiment 2. Assay sensitivity averaged 0.088 ng oPrI-I3/tube (17.6 ng/ml), and intra- and interassay variability was 1.75 and 9.8%, respectively.

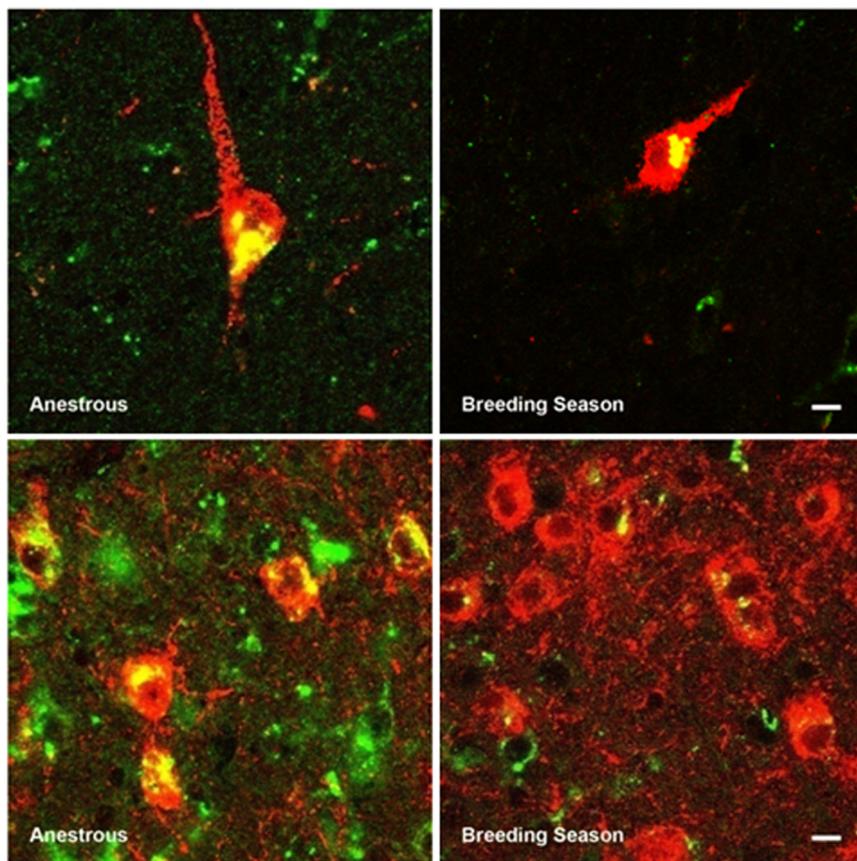
#### ICC staining

Initial analyses of single- and double-labeled GnRH and dynorphin cells were done using a Leica DM5000B fluorescence microscope (Buffalo Grove, IL) at  $\times 20$  and  $\times 40$  magnifications. The number of single- and double-labeled GnRH neurons was counted in every section processed through the POA, anterior hypothalamic area (AHA), and mediobasal hypothalamus (MBH) of each animal and the percentage of total GnRH neurons containing D2-R in each region calculated for each ewe. The number of single- and double-labeled dynorphin neurons in middle and caudal levels of the ARC was counted (two sections/each level) for each animal. Because of the density of dynorphin cells in the ARC, NeuroLucida software (MBF Biosciences, Williston, VT) was used to append multiple two-channel images, and percent colocalization of D2-R and dynorphin was determined by counting all dynorphin cells with a clear nucleus present at the focal plane of the image. In addition, as controls for non-kisspeptin-containing neurons (32), in each animal we analyzed the number of single- and double-labeled D2R/dynorphin cells throughout the POA and in the supraoptic nucleus of the hypothalamus (SON; two sections).

For illustration purposes, we captured confocal Z-stacks of 10 POA and MBH GnRH cells per area and 10 middle and caudal ARC dynorphin cells per area from OVX+E anestrus and breeding season ewes of the same material analyzed above. Confocal images were acquired at  $\times 63$  magnification using a LSM 510 META/ConfoCor2 (Carl Zeiss, Inc., New York, NY) with LSM-510 META image processing software. A 543-nm emission filter was used on a HeNe laser to view Alexa 555 fluorescent staining. A 488-nm emission filter was used on an Argon laser to visualize the Alexa-488 fluorescent staining. Z-stacks were produced with images captured at 1- $\mu\text{m}$  intervals.

#### Statistical analyses

For experiment 1, statistical comparisons were made using unpaired Student's  $t$  tests and one-way ANOVA where appropriate. For experiments 2 and 3, LH pulses were identified using previously described criteria (40): 1) the peak occurred within two samples of the previous nadir, 2) pulse amplitude exceeded assay sensitivity, and 3) the peak was 2 SD (assay variability) above the preceding and following nadirs. LH pulse frequency was analyzed by Friedman's two-way ANOVA because parametric statistics are not appropriate for this noncontinuous variable. Mean LH pulse amplitude (peak minus preceding nadir) and mean LH concentrations were calculated for each sampling period for each animal. These values were analyzed by one-way ANOVA (experiment 2) or two-way ANOVA (experiment 3) with repeated measures, using main effects of treatment (saline *vs.* Kiss1r antagonist infusion) and time (first and second sampling periods). Differences were considered statistically significant if  $P < 0.05$ .



**FIG. 1.** Confocal images (1  $\mu\text{m}$  optical sections) of GnRH cells in the MBH (top panels) and KNDy (dynorphin-ir) cells in the ARC (bottom panels) that colocalize D2-R in anestrous (left panels) and breeding season (right panels) OVX+E ewes. Top panels: GnRH-ir in red, D2-R-ir in green; bottom panels: dynorphin-ir in red, D2-R in green. Thus, yellow represents occurrence of D2-R-ir in these two cell types in all panels. Scale bar, 10  $\mu\text{m}$ .

## Results

### Experiment 1. Do dynorphin/kisspeptin and/or GnRH neurons contain D2-R, and, if so, does the expression change with season?

Western analysis of ovine tissue with the D2-R antibody revealed a single band at the expected size for D2-R (Supplemental Fig. 1). No other bands were observed, even at the lowest dilution of antibody, indicating that it did not detect any other proteins in ovine tissue. In addition, omission of D2R antibody from the dual-label procedure eliminated all D2-R staining, with GnRH or dynorphin immunoreactivity remaining unaltered (data not shown).

As expected, there were approximately twice as many GnRH cell bodies in the POA than in more posterior regions, and this distribution did not vary with season (Supplemental Fig. 2). Importantly, the number of KNDy [dynorphin immunoreactive (ir)] neurons in the middle and caudal ARC were not significantly different between anestrus ( $165 \pm 18$  and  $31 \pm 9$ , respectively) and breeding season ( $140 \pm 17$  and  $34 \pm 6$ , respectively) OVX+E ewes; there were also no seasonal differences in more rostral dynorphin-positive cell bod-

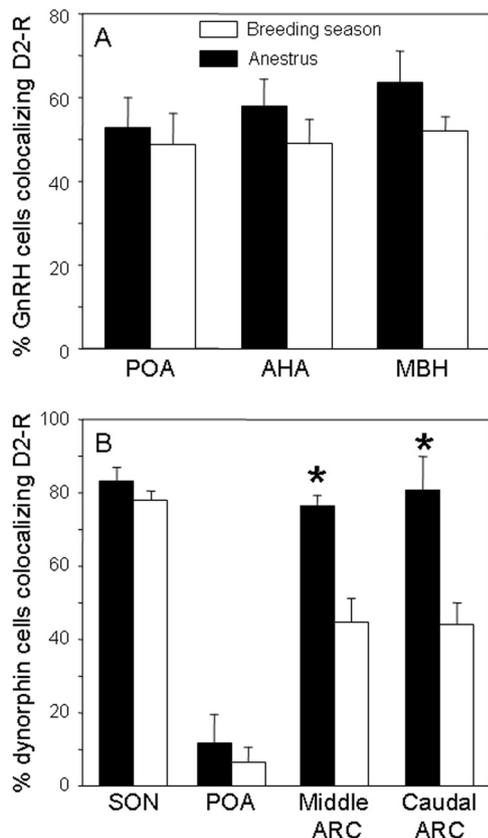
ies (Supplemental Fig. 2). D2-R was visible as punctate immunoreactivity in a subpopulation of both GnRH and KNDy neurons, and nearly all D2-R-ir particles appeared to be internalized within the cytoplasm in both cell types, regardless of region or season (Fig. 1), suggesting recent binding to endogenous DA (41). A little more than half of GnRH cell bodies contained D2-R in the POA, AHA, and MBH, and there were no significant seasonal differences in the percentage of GnRH cells expressing this receptor (Fig. 2A). Approximately 80% of ARC KNDy neurons in tissue from anestrous ewes also contained D2-R, but there was significantly less ( $P < 0.01$ , 4 degrees of freedom) colocalization in these neurons in the breeding season (Fig. 2B). For dynorphin-ir neurons in other regions, where they do not colocalize kisspeptin (32), coexpression of D2-R varied from high (SON) to low (POA) but did not change with season (Fig. 2B).

### Experiment 2. Does DA act in the ARC to hold LH pulse frequency in check in anestrous ewes?

Bilateral microinjection sites in six of the seven anestrous ewes were in, or adjacent to, the middle or caudal ARC (Fig.

3A). Microinjections in the other ewe, which produced no obvious effects on LH secretion, were dorsal to this area; data from this animal were not included in the analysis. Microinjections of the D2-R antagonist, sulpride, produced a dose-dependent increase in LH pulse frequency (Fig. 3B). The highest two doses produced three to four pulses during the 4 h after injection in most ewes (Fig. 4) and significantly ( $P < 0.05$ ) increased LH pulse frequency (Fig. 3B). An increase in pulse frequency was seen in only three of six ewes with the lowest dose of sulpride, and frequency in this group was not significantly different from controls. Mean LH concentrations showed a pattern similar to pulse frequency (Fig. 3B), but in this case the effect of dose only tended to be statistically significant ( $P = 0.056$ ,  $F = 3.15$ , 3 degrees of freedom). LH pulse amplitudes varied from a low of  $1.7 \pm 0.3$  ng/ml (in the 50 nmol group) to a high of  $2.2 \pm 0.5$  ng/ml (in the 2 nmol group) and were not significantly different among treatment groups.

Prolactin concentrations appeared to increase after the highest dose of the D2-R antagonist (Figs. 3B and 4). Statistical analysis indicated a significant main effect of treat-

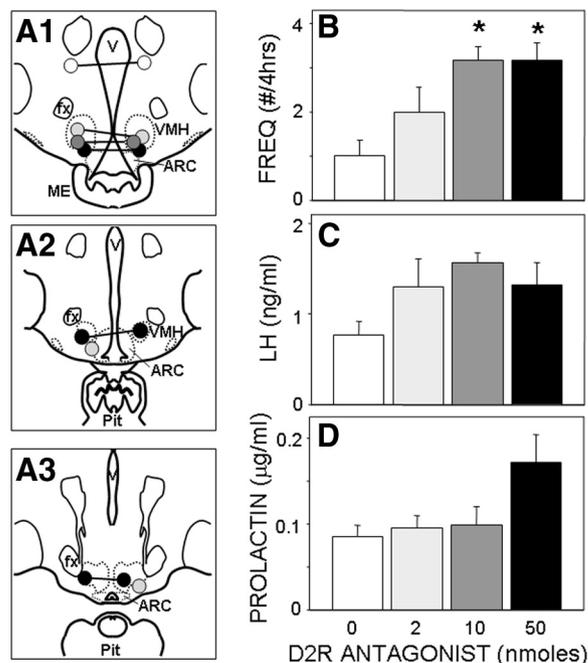


**FIG. 2.** Comparison of the percentage of GnRH-ir (A) and KNDy (dynorphin-ir) (B) cell bodies colocalizing D2-R-ir in tissue from OVX+E anestrus (solid bars) and breeding season (open bars) ewes. No seasonal differences in colocalization of D2-R with GnRH were seen in the POA, AHA, or MBH. Significantly higher ( $P < 0.01$ ) colocalization of D2-R in KNDy (dynorphin-ir) cell bodies of the middle and caudal ARC was observed in anestrus than breeding season ewes, but not in the dynorphin-positive SON or POA.

ment ( $P < 0.05$ ,  $F = 3.72$ , 3 degrees of freedom), but none of the pair-wise comparisons were different, although the comparison of the highest dose with the control tended ( $P = 0.055$ ) toward significance.

### Experiment 3. Does kisspeptin mediate the stimulatory actions of a D2-R antagonist in anestrus ewes?

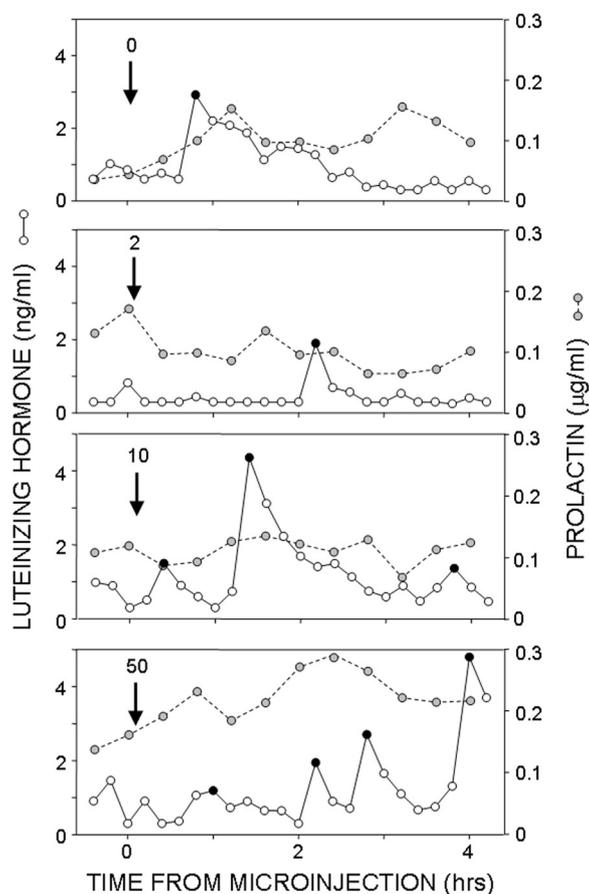
In the preliminary dose response to intracerebroventricular (icv) Kiss1r antagonist, the lowest dose (20  $\mu\text{g}/\text{h}$ ) had no obvious effect on episodic LH secretion in two OVX ewes, but the two higher doses inhibited LH secretion to some extent (Fig. 5). The middle dose (40  $\mu\text{g}/\text{h}$ ) produced a modest suppression that did not last beyond the period of treatment (Fig. 5, A and C), whereas the effects of the highest dose (60  $\mu\text{g}/\text{h}$ ) were more dramatic and lasted for several hours after the end of the infusion (Fig. 5, B and C). With both of these two effective doses, there was a delay of 20–40 min before the inhibitory effects of the antagonist were evident. Based on these data,



**FIG. 3.** Effect of localized microinjections of the D2-R antagonist, sulpiride, on LH and prolactin secretion in anestrus ewes. Panel A (left panel) depicts location of microinjections with bilateral injection in the same ewe connected with a line; in one ewe these sites were not in the same AP plane (panels A2 and A3). Data from six ewes in or near the ARC are indicated by shaded or black symbols; data from one ewe depicted by open circles in panel A1 were not used for analysis because the site was too dorsal. Panels on the right show mean ( $\pm$ SEM) LH pulse frequency (FREQ; panel B), LH concentrations (panel C), and prolactin concentrations (panel D) in the 4 h after microinjection of different doses of sulpiride. \*,  $P < 0.05$  vs. controls. fx, Fornix; ME, median eminence; Pit, pituitary stalk; V, third ventricle; VMH, ventromedial hypothalamus.

we chose the 60- $\mu\text{g}/\text{h}$  dose and began the infusion 1 h before giving the D2-R antagonist to ensure that we were effectively blocking Kiss1r in the primary experiment.

One of the six ovary-intact ewes in the main experiment was excluded from analysis because she had unexpectedly elevated LH concentrations before any treatments, and subsequently laparotomy revealed vestigial ovaries. In the other five ewes, peripheral injection of sulpiride significantly ( $P < 0.05$ , 1 degree of freedom) increased LH pulse frequency (Fig. 6) and mean LH concentrations (from  $1.2 \pm 0.4$  to  $2.8 \pm 0.6$  ng/ml) when these animals received sterile saline icv. In contrast, when these same animals received the Kiss1r antagonist icv starting 1 h before sulpiride, this D2-R antagonist had no effect on LH pulse frequency (Fig. 6) or mean LH concentrations (before:  $1.3 \pm 0.4$  ng/ml; after:  $1.8 \pm 0.9$  ng/ml). Two-way ANOVA indicated a significant ( $P < 0.05$ ,  $F = 7.77$ , 1 degree of freedom) interaction of time by treatment for mean LH concentrations. LH pulse amplitudes averaged from a low of  $3.3 \pm 0.4$  to a high of  $4.9 \pm 2.8$  ng/ml but



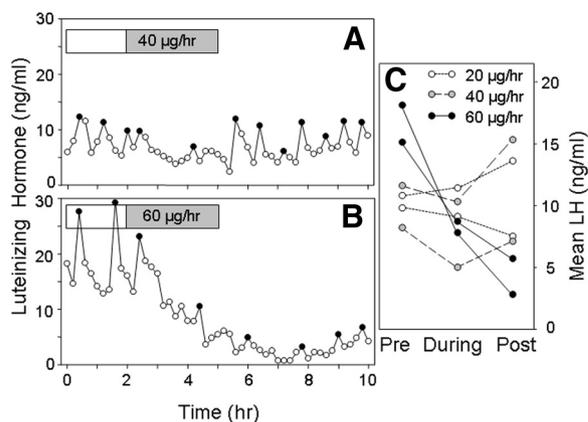
**FIG. 4.** LH pulse patterns (open circles) and concentrations of prolactin (shaded circles) in a representative ewe in response to microinjections (arrows) of four different doses of sulpiride into the ARC. Solid circles depict peaks of LH pulses.

were not analyzed statistically because of the large number of missing values (no LH pulses during a sampling period).

## Discussion

These results support the hypothesis that the inhibitory DA and stimulatory kisspeptin neurons that have been implicated in seasonal control of GnRH pulses in the ewe are hierarchical, with the DA neurons suppressing kisspeptin release to inhibit GnRH secretion during seasonal anestrus. They also support previous work implicating the ARC KNDy neurons in this system.

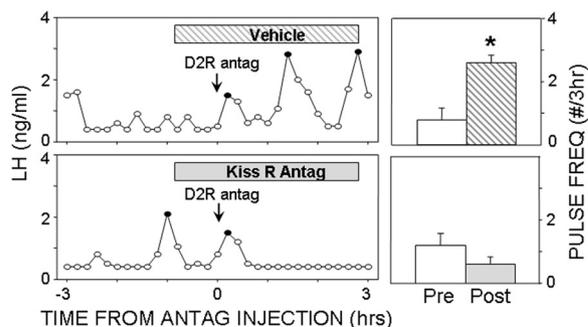
Because D2-R has been identified as the DA receptor subtype holding GnRH pulse frequency in check in ovari-intact anestrous ewes (23), this receptor must reside in any neural population that contributes to this inhibition. Therefore, the presence of D2-R in approximately 50% of GnRH neurons and up to 80% of ARC KNDy neurons is consistent with both direct and indirect inhibition of GnRH by dopaminergic input. It is unlikely, however, that GnRH cell bodies in the POA and AHA are directly in-



**FIG. 5.** Effect of infusion of different doses of the Kiss1r antagonist, p-271, into the lateral ventricle of OVX ewes. Panels on right illustrate the effects of the middle (A) and highest (B) doses of Kiss1r antagonist tested in individual ewes. Open bars, Saline infusion; shaded bar, antagonist infusion. C, Mean LH concentrations in each ewe for the 2 h before (Pre), during, and 5 h after (Post) infusion of antagonist. LH concentrations during the last 2 h of infusions were used to calculate mean LH during infusions because of the delay in effects of the antagonist (panels A and B).

hibited by A15 dopaminergic neurons because the latter do not project to these areas (15, 30). Thus, these D2-Rs are probably involved in some other aspect of GnRH secretion, possibly that regulating the preovulatory GnRH surge (42). It should be noted that previous work observed sparse DA input to ovine GnRH neurons (43, 44), with only about 15% of POA GnRH neurons receiving DA-containing close contacts (43). However, the high degree of D2-R internalization we observed suggests that almost 50% of them receive DA input, so the former data (43) may have been an underestimation of these afferents.

Because A15 neurons project to the MBH and median eminence (15, 30), they may directly inhibit GnRH pulses at GnRH cell bodies in the MBH or GnRH terminals in the median eminence (see below). The presence of D2-R in



**FIG. 6.** Kiss1r antagonist blocks actions of D2-R antagonist in ovari-intact anestrous ewes. Left panel, LH pulse patterns before and after injection of D2-R antagonist (arrows) in a ewe receiving saline (top panel) or Kiss1r antagonist (bottom panel) icv. Bars depict period of infusion into the lateral ventricle. Right panel, Mean LH pulse frequency (FREQ) pre (PRE) and post (POST) injection of D2-R antagonist with control (top panel) and Kiss1r antagonist (bottom panel) treatments. \*,  $P < 0.05$  vs. pre-treatment.

ARC KNDy neurons, and the high percentage of KNDy neurons in which this receptor is internalized, are consistent with this population mediating the inhibitory actions of DA in anestrus ewes. Moreover, the 2-fold increase in percentage of these KNDy neurons containing D2-R in OVX+E anestrus ewes raises the possibility that these neurons are particularly sensitive to dopaminergic inhibition at this time of year. It should be noted that an early study using a D2-R agonist failed to detect seasonal differences in responsiveness, but only one dose was tested (45), so a more complete dose response may be warranted.

The ability of local administration of a D2-R antagonist near, or into, the ARC to stimulate LH pulse frequency also supports the hypothesis that kisspeptin from KNDy neurons is an important mediator of dopaminergic inhibition of GnRH. These results are consistent with previous reports that local administration of a D2-R antagonist via microimplants (46) and microdialysis (24) to the MBH and median eminence, respectively, increased LH pulse frequency in anestrus ewes. Interpretation of microinjection studies is limited to some extent because the volume of tissue affected is difficult to assess. Thus, the antagonist could be acting in the ventromedial hypothalamus or median eminence. There is pharmacological evidence that D2-Rs in the ventromedial hypothalamus are important for control of estrous behavior in ewes (47), but they are unlikely to mediate the effects of A15 neurons because no A15 efferents project to this area (15, 30). An action in the median eminence is more likely in light of A15 projections to this region (15, 30). Therefore, we addressed this issue by monitoring the effects of this D2-R antagonist on prolactin concentrations because the administration of sulpiride to the median eminence was more effective in stimulating prolactin than LH secretion (24). Thus, the observation that LH pulse frequency was increased after microinjection of a dose (10 nmol) that had no effect on prolactin concentrations (Fig. 3) argues that, at least at this dose, little sulpiride was reaching the median eminence and that the ARC is the most likely site of action. The lack of effect of sulpiride on prolactin concentrations also argues against a local effect of this antagonist on A12 DA neurons in the ARC, which are thought to regulate prolactin secretion (48).

The strongest evidence that DA inhibits GnRH pulse frequency by suppressing kisspeptin release comes from the observation that the stimulatory effects of a D2-R antagonist were blocked by icv administration of the Kiss1r antagonist. These data are consistent with previous work in sheep demonstrating that a similar Kiss1r antagonist inhibited episodic LH secretion in OVX ewes (35) and demonstrate that kisspeptin release mediates the increase in LH secretion resulting from the removal of DA inhibi-

tion. These results do not identify which kisspeptin neurons are involved, but the effects of D2-R antagonist in (46, this study) or near (24) the ARC point to this population of kisspeptin neurons. In contrast, microimplants of a D2-R antagonist in the POA (46) had no effect on LH secretion in anestrus ewes. This hypothesis is consistent with previous evidence in the ewe that kisspeptin expression in the ARC is lower in OVX+E ewes during anestrus than in the breeding season (16); in contrast, E<sub>2</sub> stimulated kisspeptin expression in the POA population in both anestrus and the breeding season (16).

This proposed role for ARC kisspeptin neurons is also consistent with data in male Syrian hamsters (*Mesocricetus auratus*), in which short-day photoperiod (SD) inhibits reproductive function (7). In these hamsters, kisspeptin expression in the ARC is controlled by melatonin (7, 49) so that it decreases in SD, independent of circulating testosterone, and exogenous kisspeptin restores testicular size in SD-suppressed males (49). The role of AVPV kisspeptin neurons in this species is unclear because they are difficult to detect, even in long-day photoperiod hamsters (49, 50); there is one report that their number decreases in SD (50), but the mechanisms and significance of these changes remain to be determined. In contrast, in Siberian hamsters (*Phodopus sungorus*), kisspeptin expression in the ARC is elevated in SD-suppressed males, but expression in the AVPV is inhibited (7, 51). It is unlikely, however, that changes in AVPV kisspeptin neural activity are critical to seasonal reproduction in these hamsters because exogenous kisspeptin does not induce testicular growth during SD exposure (52) and kisspeptin expression in the AVPV appears to be driven largely by changes in circulating testosterone (53). Thus, in sheep and Syrian, but not Siberian, hamsters, ARC kisspeptin neurons appear to play a key role in seasonal changes in the control of reproduction.

Although this report supports the hypothesis that the negative feedback actions of E<sub>2</sub> in the anestrus ewe are conveyed via A15 dopaminergic neurons that suppress kisspeptin release from ARC neurons and thereby inhibit GnRH pulse frequency, they do not exclude other parallel pathways. For example, A15 DA neurons do project to the median eminence (15, 30) in which they may synapse on GnRH terminals (54). The presence of D2-Rs in 50% of GnRH cell bodies indicates that this receptor is likely transported to and found on GnRH terminals, and direct application of a D2-R antagonist to the median eminence increased LH pulse frequency in anestrus ewes (24). Given the importance of seasonal breeding to the survival of these species, it would not be surprising that multiple pathways had evolved to limit fertility to a specific time of year.

In summary, these experiments demonstrate that DA acts in the ARC to hold LH pulse frequency in check, that kisspeptin neurons in this area contain D2-R, and that kisspeptin is necessary for the increase in LH pulse frequency that occurs when D2-Rs are blocked with systemic administration of an antagonist. Thus, taken together, these data provide strong support for the hypothesis that the dopaminergic suppression of LH pulse frequency in ovary-intact anestrous ewes occurs by inhibiting stimulatory kisspeptin afferents to GnRH neurons.

## Acknowledgments

We thank Heather Bungard and Jennifer Lydon (West Virginia University Food Animal Research Facility) for the care of animals and Paul Harton for his technical assistance in sectioning tissue. We also thank Dr. Al Parlow and the National Hormone and Peptide Program (Torrance, CA) for the reagents used to measure LH and prolactin.

Address all correspondence and requests for reprints to: Robert L. Goodman, Department of Physiology and Pharmacology, P.O. Box 9229, West Virginia University, Morgantown, West Virginia, 26506. E-mail: rgoodman@hsc.wvu.edu.

This work was supported by National Institutes of Health Grant R01 HD017864.

Disclosure Summary: The authors have nothing to disclose.

## References

- Karsch FJ, Bittman EL, Foster DL, Goodman RL, Legan SJ, Robinson JE 1984 Neuroendocrine basis of seasonal reproduction. *Recent Prog Horm Res* 40:185–232
- Malpaux B 2006 Seasonal regulation of reproduction in mammals. In: Neill JD, ed. *Knobil and Neill's physiology of reproduction*. Vol 2. 3rd ed. Amsterdam: Elsevier; 2231–2282
- Bartness TJ, Goldman BD, Bittman EL 1991 SCN lesions block responses to systemic melatonin infusions in Siberian hamsters. *Am J Physiol* 260:R102–R112
- Maywood ES, Hastings MH 1995 Lesions of the iodomelatonin-binding sites of the mediobasal hypothalamus spare the lactotropic, but block the gonadotropic response of male Syrian hamsters to short photoperiod and to melatonin. *Endocrinology* 136:144–153
- Malpaux B, Daveau A, Maurice-Mandon F, Duarte G, Chemineau P 1998 Evidence that melatonin acts in the premammillary hypothalamic area to control reproduction in the ewe: presence of binding sites and stimulation of luteinizing hormone secretion by *in situ* microimplant delivery. *Endocrinology* 139:1508–1516
- Hanon EA, Lincoln GA, Fustin JM, Dardente H, Masson-Pévet M, Morgan PJ, Hazlerigg DG 2008 Ancestral TSH mechanism signals summer in a photoperiodic mammal. *Curr Biol* 18:1147–1152
- Revel FG, Masson-Pévet M, Pévet P, Mikkelsen JD, Simonneaux V 2009 Melatonin controls seasonal breeding by a network of hypothalamic targets. *Neuroendocrinology* 90:1–14
- Clarke IJ, Smith JT, Caraty A, Goodman RL, Lehman MN 2009 Kisspeptin and seasonality in sheep. *Peptides* 30:154–163
- de Roux N, Genin E, Carel JC, Matsuda F, Chaussain JL, Milgram E 2003 Hypogonadotropic hypogonadism due to loss of function of the Kiss1-derived peptide receptor GPR54. *Proc Natl Acad Sci USA* 100:10972–10976
- Seminara SB, Messager S, Chatzidaki EE, Thresher RR, Acierno Jr JS, Shagoury JK, Bo-Abbas Y, Kuohung W, Schwinof KM, Hendrick AG, Zahn D, Dixon J, Kaiser UB, Slaugenhaupt SA, Gusella JF, O'Rahilly S, Carlton MB, Crowley Jr WF, Aparicio SA, Colledge WH 2003 The GPR54 gene as a regulator of puberty. *N Engl J Med* 349:1614–1627
- García-Galiano D, Pinilla L, Tena-Sempere M 2012 Sex steroids and the control of the Kiss1 system: developmental roles and major regulatory actions. *J Neuroendocrinol* 24:22–33
- Smith JT, Popa SM, Clifton DK, Hoffman GE, Steiner RA 2006 Kiss1 neurons in the forebrain as central processors for generating the preovulatory luteinizing hormone surge. *J Neurosci* 26:6687–6694
- Oakley AE, Clifton DK, Steiner RA 2009 Kisspeptin signaling in the brain. *Endocr Rev* 30:713–743
- Barrel GK, Moenter SM, Caraty A, Karsch FJ 1992 Seasonal changes of gonadotropin-releasing hormone secretion in the ewe. *Biol Reprod* 46:1130–1135
- Goodman RL, Jansen HT, Billings HJ, Coolen LM, Lehman MN 2010 Neural systems mediating seasonal breeding in the ewe. *J Neuroendocrinol* 22:674–681
- Smith JT, Coolen LM, Kriegsfeld LJ, Sari IP, Jaafarzadehshirazi MR, Maltby M, Bateman K, Goodman RL, Tilbrook AJ, Ubuka T, Bentley GE, Clarke IJ, Lehman MN 2008 Variation in kisspeptin and RFamide-related peptide (RFRP) expression and terminal connections to gonadotropin-releasing hormone neurons in the brain: a novel medium for seasonal breeding in the sheep. *Endocrinology* 149:5770–5782
- Chalivoix S, Bagnolini A, Caraty A, Cognié J, Malpaux B, Dufourny L 2010 Effects of photoperiod on kisspeptin neuronal populations of the ewe diencephalon in connection with reproductive function. *J Neuroendocrinol* 22:110–118
- Caraty A, Smith JT, Lomet D, Ben Saïd S, Morrissey A, Cognie J, Doughton B, Baril G, Briant C, Clarke IJ 2007 Kisspeptin synchronizes preovulatory surges in cyclical ewes and causes ovulation in seasonally acyclic ewes. *Endocrinology* 148:5258–5267
- Sébert ME, Lomet D, Saïd SB, Monget P, Briant C, Scaramuzzi RJ, Caraty A 2010 Insights into the mechanism by which kisspeptin stimulates a preovulatory LH surge and ovulation in seasonally acyclic ewes: potential role of estradiol. *Domest Anim Endocrinol* 38:289–298
- Lehman MN, Durham DM, Jansen HT, Adrian B, Goodman RL 1996 Dopaminergic A14/A15 neurons are activated during estradiol negative feedback in anestrous, but not breeding season, ewes. *Endocrinology* 137:4443–4450
- Thiéry JC, Martin GB, Tillet Y, Caldani M, Quentin M, Jamain C, Ravault JP 1989 Role of hypothalamic catecholamines in the regulation of luteinizing hormone and prolactin secretion in the ewe during seasonal anestrus. *Neuroendocrinology* 49:80–87
- Havern RL, Whisnant CS, Goodman RL 1994 Dopaminergic structures in the ovine hypothalamus mediating estradiol negative feedback in anestrous ewes. *Endocrinology* 134:1905–1914
- Curler JD, Naylor AM, McNeilly AS 1991 Evaluation of a possible role for the dopamine D<sub>1</sub> and D<sub>2</sub> receptors in the steroid-dependent suppression of luteinizing hormone secretion in the seasonally anoestrous ewe. *J Neuroendocrinol* 3:387–391
- Bertrand F, Thiery J, Picard S, Malpaux B 1999 Implication of D2-like dopaminergic receptors in the median eminence during the establishment of long-day inhibition of LH secretion in the ewe. *J Endocrinol* 163:243–254
- Lehman MN, Karsch FJ 1993 Do gonadotropin-releasing hormone, tyrosine hydroxylase-, and  $\beta$ -endorphin-immunoreactive neurons contain estrogen receptors? A double-label immunocytochemical study in the Suffolk ewe. *Endocrinology* 133:887–895
- Skinner DC, Herbison AE 1997 Effects of photoperiod on estrogen

- receptor, tyrosine hydroxylase, neuropeptide Y, and  $\beta$ -endorphin immunoreactivity in the ewe hypothalamus. *Endocrinology* 138:2585–2595
27. Anderson GM, Connors JM, Hardy SL, Valent M, Goodman RL 2001 Oestradiol microimplants in the ventromedial preoptic area inhibit secretion of luteinising hormone via dopaminergic neurons in anoestrous ewes. *J Neuroendocrinol* 13:1051–1058
  28. Gallegos-Sánchez J, Delaleu B, Caraty A, Malpoux B, Thiéry JC 1997 Estradiol acts locally within the retrochiasmatic area to inhibit pulsatile luteinizing hormone release in the female sheep during anestrus. *Biol Reprod* 56:1544–1549
  29. Hardy SL, Anderson GM, Valent M, Connors JM, Goodman RL 2003 Evidence that estrogen receptor  $\alpha$ , but not  $\beta$ , mediates seasonal changes in the response of the ovine retrochiasmatic area to estradiol. *Biol Reprod* 68:846–852
  30. Gayard V, Thiéry JC, Thibault J, Tillet Y 1995 Efferent projections from the retrochiasmatic area to the median eminence and to the pars nervosa of the hypophysis with special reference to the A15 dopaminergic cell group in the sheep. *Cell Tissue Res* 281:561–567
  31. Goodman RL, Lehman MN, Smith JT, Coolen LM, de Oliveira CV, Jafarzadehshirazi MR, Pereira A, Iqbal J, Caraty A, Ciofi P, Clarke IJ 2007 Kisspeptin neurons in the arcuate nucleus of the ewe also express dynorphin A and neurokinin B. *Endocrinology* 148:5752–5760
  32. Lehman MN, Coolen LM, Goodman RL 2010 KNDy (kisspeptin/neurokinin B/dynorphin) cells of the arcuate nucleus: a central node in the control of GnRH secretion. *Endocrinology* 151:3479–3489
  33. Foradori CD, Amstalden M, Coolen LM, Singh SR, McManus CJ, Handa RJ, Goodman RL, Lehman MN 2007 Orphanin FQ: evidence for a role in the control of the reproductive neuroendocrine axis. *Endocrinology* 148:4993–5001
  34. Foradori CD, Coolen LM, Fitzgerald ME, Skinner DC, Goodman RL, Lehman MN 2002 Colocalization of progesterone receptors in the parvocellular dynorphin neurons of the ovine preoptic area and hypothalamus. *Endocrinology* 143:4366–4374
  35. Roseweir AK, Kauffman AS, Smith JT, Guerriero KA, Morgan K, Pielecka-Fortuna J, Pineda R, Gottsch ML, Tena-Sempere M, Moenter SM, Terasawa E, Clarke IJ, Steiner RA, Millar RP 2009 Discovery of potent kisspeptin antagonists delineate physiological mechanisms of gonadotropin regulation. *J Neurosci* 29:3920–3929
  36. Pineda R, García-Galiano D, Roseweir A, Romero M, Sanchez-Garrido MA, Ruiz-Pino F, Morgan K, Pinilla L, Millar RP, Tena-Sempere M 2010 Critical roles of kisspeptins in female puberty and preovulatory gonadotropin surges as revealed by a novel antagonist. *Endocrinology* 151:722–730
  37. Smith JT, Li Q, Sing Yap KS, Shahab M, Roseweir AK, Millar RP, Clarke IJ 2011 Kisspeptin is essential for the full preovulatory LH surge and stimulates GnRH release from the isolated ovine median eminence. *Endocrinology* 152:1001–1012
  38. Goodman RL, Coolen LM, Anderson GM, Hardy SL, Valent M, Connors JM, Fitzgerald ME, Lehman MN 2004 Evidence that dynorphin plays a major role in mediating progesterone negative feedback on gonadotropin-releasing hormone neurons in sheep. *Endocrinology* 145:2959–2967
  39. Meyer SL, Goodman RL 1985 Neurotransmitters involved in mediating the steroid-dependent suppression of pulsatile luteinizing hormone secretion in anoestrous ewes: effects of receptor antagonists. *Endocrinology* 116:2054–2061
  40. Goodman RL, Karsch FJ 1980 Pulsatile secretion of luteinizing hormone: differential suppression by ovarian steroids. *Endocrinology* 107:1286–1290
  41. Yao WD, Spealman RD, Zhang J 2008 Dopaminergic signaling in dendritic spines. *Biochem Pharmacol* 75:2055–2069
  42. Goodman RL, Inskoop EK 2006 Control of the ovarian cycle of the sheep. In: Neill JD, ed. *Knobil and Neill's physiology of reproduction*. Vol 2. 3rd ed. Amsterdam: Elsevier; 2389–2447
  43. Pompolo S, Pereira A, Kaneko T, Clarke IJ 2003 Seasonal changes in the inputs to gonadotropin-releasing hormone neurones in the ewe brain: an assessment by conventional fluorescence and confocal microscopy. *J Neuroendocrinol* 15:538–545
  44. Jansen HT, Cutter C, Hardy S, Lehman MN, Goodman RL 2003 Seasonal plasticity in the GnRH system of the ewe: changes in identified GnRH inputs and in glial association. *Endocrinology* 144:3663–3676
  45. Meyer SL, Goodman RL 1986 Separate neural systems mediate the steroid-dependent and steroid-independent suppression of tonic luteinizing hormone secretion in the anoestrous ewe. *Biol Reprod* 35:562–571
  46. Havern RL, Whisnant CS, Goodman RL 1991 Hypothalamic sites of catecholamine inhibition of luteinizing hormone in the anoestrous ewe. *Biol Reprod* 44:476–482
  47. Fabre-Nys C, Chesneau D, de la Riva C, Hinton MR, Locatelli A, Ohkura S, Kendrick KM 2003 Biphasic role of dopamine on female sexual behaviour via D2 receptors in the mediobasal hypothalamus. *Neuropharmacology* 44:354–366
  48. Anderson ST, Curlew JD 1998 PACAP stimulates dopamine neuronal activity in the medial basal hypothalamus and inhibits prolactin. *Brain Res* 790:343–346
  49. Revel FG, Saboureau M, Masson-Pévet M, Pévet P, Mikkelsen JD, Simonneaux V 2006 Kisspeptin mediates the photoperiodic control of reproduction in hamsters. *Curr Biol* 16:1730–1735
  50. Revel FG, Ansel L, Klosen P, Saboureau M, Pévet P, Mikkelsen JD, Simonneaux V 2007 Kisspeptin: a key link to seasonal breeding. *Rev Endocr Metab Disord* 8:57–65
  51. Greives TJ, Mason AO, Scotti MA, Levine J, Ketterson ED, Kriegsfeld LJ, Demas GE 2007 Environmental control of kisspeptin: implications for seasonal reproduction. *Endocrinology* 148:1158–1166
  52. Greives TJ, Kriegsfeld LJ, Demas GE 2008 Exogenous kisspeptin does not alter photoperiod-induced gonadal regression in Siberian hamsters (*Phodopus sungorus*). *Gen Comp Endocrinol* 156:552–558
  53. Greives TJ, Humber SA, Goldstein AN, Scotti MA, Demas GE, Kriegsfeld LJ 2008 Photoperiod and testosterone interact to drive seasonal changes in kisspeptin expression in Siberian hamsters (*Phodopus sungorus*). *J Neuroendocrinol* 20:1339–1347
  54. Kuljis RO, Advis JP 1989 Immunocytochemical and physiological evidence of a synapse between dopamine- and luteinizing hormone releasing hormone-containing neurons in the ewe median eminence. *Endocrinology* 124:1579–1581