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)**

Reproductive 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.

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Abbreviations: AHA, Anterior hypothalamic area; ARC, arcuate nucleus; AVPV, anteroventral periventricular nucleus; DA, dopaminergic; D2-R, dopamine receptor; E_2, 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 anestrous (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 anestrous 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 anestrous 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 anestrous 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 anestrous 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 anestrous (May) and breeding season (November) ewes. Experiments 2 and 3 were performed from the beginning of June through early July in anestrous 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 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% isofluorane as anesthesia. Ovaries were removed via midventral laporatomy 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% $NaNO_3$. 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-μm-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 μm 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_2 (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 extracted. 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_2O_2 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 antimouse (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_2O_2 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 anestrous 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 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 μl/h) was infused into the lateral ventricle for 0–2 h in all six ewes, followed by p-271 (20, 40, or 60 μg/h, n = 2/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 anestrous ewes?

A chronic16-gauge needle was implanted into the right lateral ventricle of six ovary-intact anestrous ewes in early June. Approximately 2 weeks later, sulpiride [12 mg/ml (35 μmol/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 μl/h, n = 3) or Kiss1r antagonist (60 μg/h, n = 3) was infused from h 2–6, and at h 3, all six ewes were given an im injection of sulpiride [1.2 mg/kg (3.5 μmol/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 crossover 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 μl) 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-μl aliquots of every other sample collected during experiment 2. Assay sensitivity averaged 0.088 ng oPrl-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 ×20 and ×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 anestrous and breeding season ewes of the same material analyzed above. Confocal images were acquired at ×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-μm 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 $P < 0.05$.

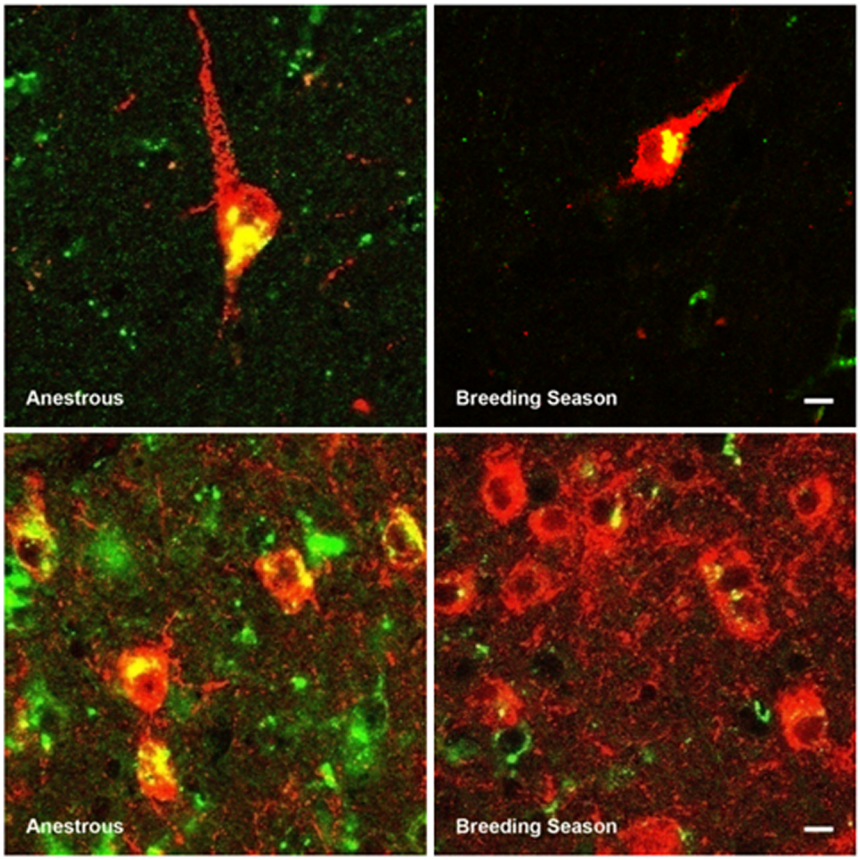

FIG. 1. Confocal images (1 μm optical sections) of GnRH cells in the MBH (*top panels*) and KNDy (dynorphin-ir) cells in the ARC (*bottom panels*) that colocalize D2-R in anestrous (*left panels*) and breeding season (*right panels*) OVX+E ewes. *Top panels*: GnRH-ir in *red*, D2-R-ir in *green*; *bottom 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 μm.

Results

Experiment 1. Do dynorphin/kisspetin 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 bodies (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-

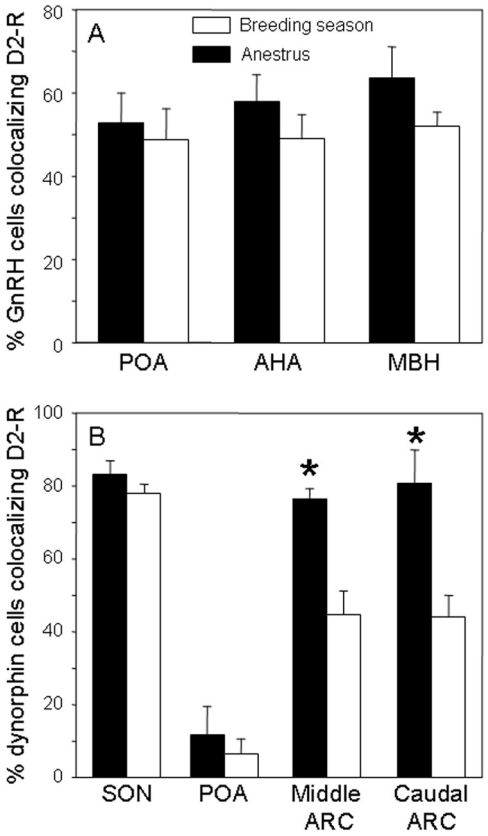

FIG. 2. Comparison of the percentage of GnRH-ir (A) and KNDy (dynorphin-ir) (B) cell bodies colocalizing D2-R-ir in tissue from OVX+E anestrous (*solid bars*) and breeding season (*open bars*) ewes. No seasonal differences in colocalization of D2-R with GnRH were seen in the POA, AHA, or MBH. Significantly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

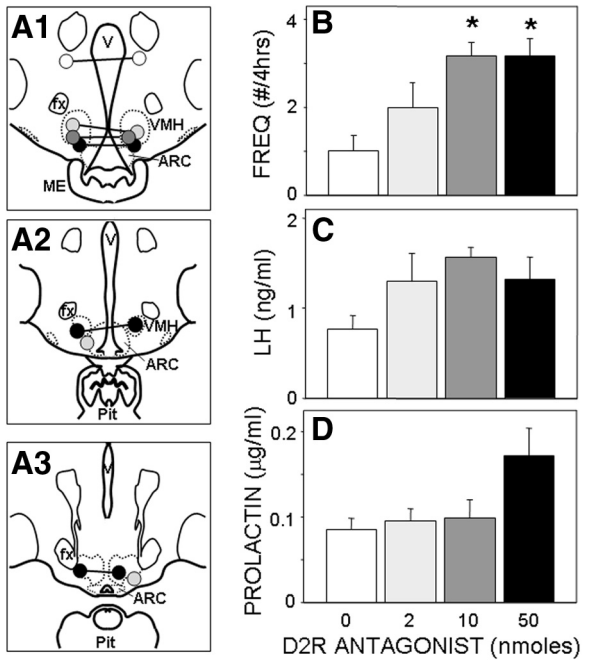

FIG. 3. Effect of localized microinjections of the D2-R antagonist, sulpiride, on LH and prolactin secretion in anestrous ewes. Panel A (*left panel*) depicts location of microinjections with bilateral injection in the same ewe connected with a *line*; in one ewe these sites were not in the same AP plane (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 (±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.

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 anestrous ewes?

In the preliminary dose response to intracerebroventricular (icv) Kiss1r antagonist, the lowest dose (20 μg/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 μg/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 μg/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, we chose the 60-μg/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 laporatomy 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 ± 0.4 to 2.8 ± 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 ± 0.4 ng/ml; after: 1.8 ± 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 ± 0.4 to a high of 4.9 ± 2.8 ng/ml but

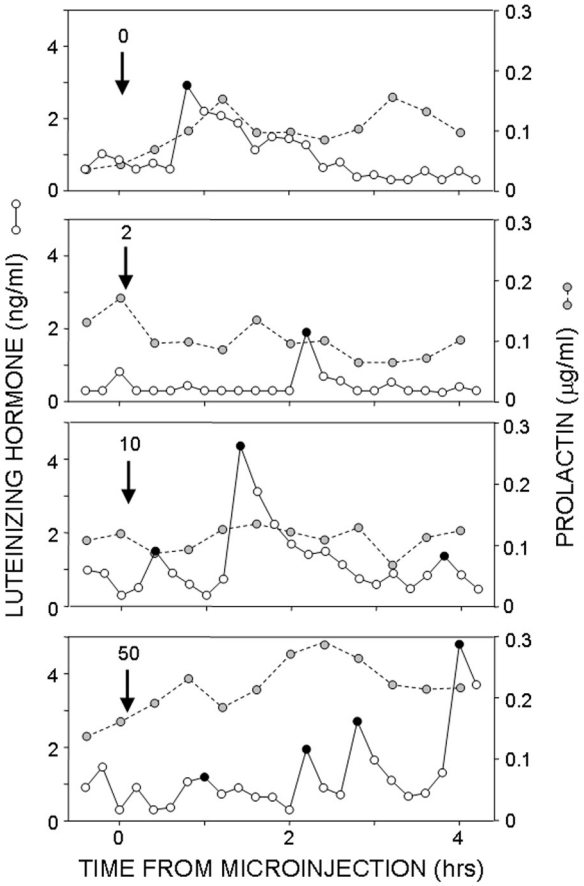

FIG. 4. LH pulse patterns (*open circles*) and concentrations of prolactin (*shaded circles*) in a representative ewe in response to microinjections (*arrows*) of four different doses of sulpiride into the ARC. *Solid circles* depict peaks of LH pulses.

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 ovary-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 inhibited 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.

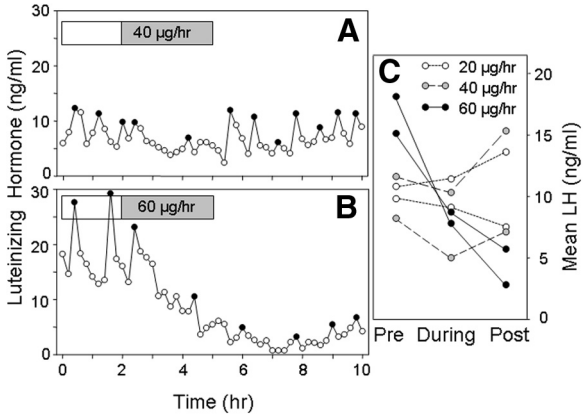

FIG. 5. Effect of infusion of different doses of the Kiss1r antagonist, p-271, into the lateral ventricle of OVX ewes. *Panels on right* illustrate the effects of the middle (A) and highest (B) doses of Kiss1r antagonist tested in individual ewes. *Open bars*, Saline infusion; *shaded bar*, antagonist 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).

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

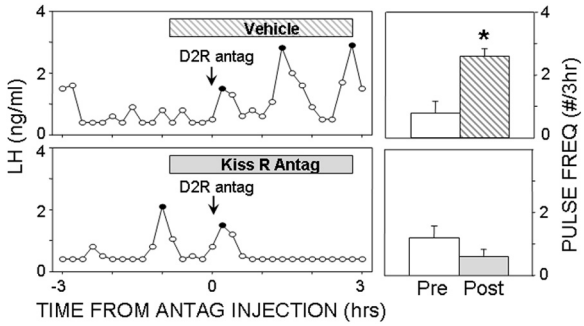

FIG. 6. Kiss1r antagonist blocks actions of D2-R antagonist in ovary-intact anestrous ewes *Left panel*, LH pulse patterns before and after im injection of D2-R antagonist (*arrows*) in a ewe receiving saline (*top panel*) or Kiss1r antagonist (*bottom panel*) icv. *Bars* depict period of infusion into 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 anestrous ewes. Moreover, the 2-fold increase in percentage of these KNDy neurons containing D2-R in OVX+E anestrous 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 anestrous 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 inhibition. 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 anestrous ewes. This hypothesis is consistent with previous evidence in the ewe that kisspeptin expression in the ARC is lower in OVX+E ewes during anestrous than in the breeding season (16); in contrast, E_2 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_2 in the anestrous 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 anestrous 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.

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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.

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