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Kisspeptin Neurons in the Arcuate Nucleus of the Ewe Express Both Dynorphin A and Neurokinin B

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Kisspeptin is a potent stimulator of GnRH secretion that has been implicated in the feedback actions of ovarian steroids. In ewes, the majority of hypothalamic kisspeptin neurons are found in the arcuate nucleus (ARC), with a smaller population located in the preoptic area. Most arcuate kisspeptin neurons express estrogen receptor-α, as do a set of arcuate neurons that contain both dynorphin and neurokinin B (NKB), suggesting that all three neuropeptides are colocalized in the same cells. In this study we tested this hypothesis using dual immunocytochemistry and also determined if kisspeptin neurons contain MSH or agouti-related peptide. To assess colocalization of kisspeptin and dynorphin, we used paraformaldehyde-fixed tissue from estrogen-treated ovariectomized ewes in the breeding season (n = 5). Almost all ARC, but no preoptic area, kisspeptin neurons contained dynorphin. Similarly, almost all ARC dynorphin neurons contained kisspeptin. In experiment 2 we examined colocalization of kisspeptin and NKB in picric-acid fixed tissue collected from ovary intact ewes (n = 9). Over three quarters of ARC kisspeptin neurons also expressed NKB, and a similar percentage of NKB neurons contained kisspeptin. In contrast, no kisspeptin neurons stained for MSH or agouti-related peptide. These data demonstrate that, in the ewe, a high percentage of ARC kisspeptin neurons also produce dynorphin and NKB, and we propose that a single subpopulation of ARC neurons contains all three neuropeptides. Because virtually all of these neurons express estrogen and progesterone receptors, they are likely to relay the feedback effects of these steroids to GnRH neurons to regulate reproductive function. (*Endocrinology* 148: 5752–5760, 2007)

SINCE KISSPEPTIN burst onto the reproductive neuroendocrine scene in 2003, a consensus has developed that this neuropeptide is a key regulator of GnRH, and, thus LH, secretion (1–3). The *Kiss 1* gene encodes a large (132–145 amino acids) precursor (3) that contributes to a family of smaller peptides, ranging from 10–54 amino acids, which act via a G-coupled protein receptor (GPR54) to stimulate GnRH and LH release in rodents (4–7), sheep (8), monkeys (9), and humans (10). Mutations of GPR54 prevent the onset of puberty in humans (11, 12) and mice (13). Kisspeptin has been implicated in the feedback actions of ovarian steroids (see below), and kisspeptin expression correlates with changes in fertility associated with puberty (2, 14, 15), undernutrition (16), and seasonal breeding (17–19).

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Abbreviations: AGRP, Agouti-related peptide; ARC, arcuate nucleus; AVPV, anteroventral periventricular nucleus; E_2, estradiol; ER, estrogen receptor; GnIH, gonadotropin-inhibiting hormone; GPR, G-coupled protein receptor; ICC, immunocytochemistry; NFF, neuropeptide FF; NGS, normal goat serum; NKB, neurokinin B; PB, phosphate buffer; POA, preoptic area; PR, progesterone receptor; PrRP, prolactin-releasing peptide; RT, room temperature; TBS, Tris-buffered saline; TSA, tyramide amplification solution.

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In mice and rats, two populations of kisspeptin-containing neurons have been identified: a rostral group centered in the anteroventral periventricular nucleus (AVPV) and a more caudal group primarily found in the arcuate nucleus (ARC) (1, 4, 20–22). AVPV kisspeptin neurons appear to mediate the positive feedback action of estradiol (E_2) because: 1) antisera to kisspeptin blocks the preovulatory LH surge (23); 2) E_2 stimulates expression of *Kiss 1* in the AVPV (21, 22); 3) both *Kiss 1* mRNA and Fos antigen increase in these cells at the preovulatory LH surge (24); and 4) these neurons are sexually differentiated, with more kisspeptin-containing neurons in females than males (25, 26). In contrast, the ARC subpopulation of kisspeptin neurons has been proposed to mediate the negative feedback actions of steroids in rodents, largely because E_2 and testosterone inhibit expression of *Kiss 1* mRNA in the ARC (1, 7, 21, 22).

A qualitatively similar distribution of kisspeptin-containing neurons has been reported in sheep (17, 27), monkeys (14, 28), and humans (28), but there is a greater abundance of these cells in the ARC in these species, with a smaller population (sheep) or a few scattered cells (humans) in the preoptic area (POA). This quantitative difference may relate to the site of E_2 positive feedback, which occurs in the medial basal hypothalamus in sheep (29) and primates (30). An increase in *Kiss 1* mRNA levels is observed in a subpopula-

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tion of these ARC neurons just before the LH surge in ewes (31), suggesting a possible role in the positive feedback actions of E_2 in this species. As in rodents, ovariectomy of sheep (17) and primates (28), or menopause in women (28), increases *Kiss 1* gene expression in the ARC, so these neurons may also play a role in steroid negative feedback.

Although there is considerable evidence that kisspeptin neurons are important regulators of GnRH secretion, little is known about their morphological characteristics. Most kisspeptin neurons express α-estrogen receptors (ER) (21, 22, 24, 27) and progesterone receptors (PR) (17), consistent with their postulated role as mediators of steroid feedback. Some (~40%) of the ARC kisspeptin neurons in the mouse also express the leptin receptor (32). Recently, the possibility of colocalization of dopamine with kisspeptin in the rat AVPV was examined, but less than 5% of these kisspeptin neurons also stained for tyrosine hydroxylase (26). We have identified a group of neurons in the ovine ARC that produce both neurokinin B (NKB) and dynorphin A (33), most of which also express ERα and PR (34, 35). The high percentage of kisspeptin neurons expressing these steroid receptors in the ovine ARC raises the possibility that these two populations of neurons are the same. Alternatively, because kisspeptin has been implicated in the nutritional regulation of reproductive function (16), and many kisspeptin neurons contain leptin receptors (32), this neuropeptide might be colocalized with either γ-MSH or agouti-related peptide (AGRP). Neurons producing γ-MSH or AGRP in the ovine ARC are known to respond to metabolic signals. In the present study, we tested these alternate hypotheses using dual immunocytochemistry (ICC) for kisspeptin and each of the other four neurotransmitters.

Materials and Methods

Animals

Adult Suffolk crossbred (experiment 1) and Corriedale (experiment 2) ewes were maintained in a sheltered facility during the breeding season and moved indoors 1–2 d before experimental work. Animals were fed a maintenance ration daily and had free access to water. For experiment 1, ewes (n = 5) were ovariectomized, and a 3-cm long SILASTIC brand implant (Dow Corning, Corp., Midland, MI) containing E_2 inserted sc 2 wk before tissue was collected. Ovaries were removed via a midventral incision using sterile techniques with animals anesthetized with 1–3% halothane in nitrous oxide: oxygen gas. For experiment 2, estrous cycles were synchronized using a 125-μg im injection of the synthetic luteolysin, cloprostenol (Estrumate; Jurox, Silverwater, New South Wales, Australia), and tissue collected (n = 3 per group) during the midluteal phase (d 8–10 after estrus), the follicular phase (24 h after injection of cloprostenol), and estrus (1 h after the start of estrous behavior) for a study that has been published (31). All animal work was approved by the appropriate institutional Animal Care and Use Committee.

Tissue collection

Tissue was collected and processed as previously described (33, 36). Briefly, ewes were killed with an overdose of sodium pentobarbital, and their heads were perfused with either 6 liters of 4% paraformaldehyde in 0.1 M phosphate buffer (PB) (pH 7.4) containing heparin (10 U/ml) and $NaNO_3$ (experiment 1), or with 2 liters of heparinized saline (12.5 U/ml), followed by 2 liters of 4% paraformaldehyde plus 15% picric acid in PB, and then 1 liter of the same fixative containing 20% sucrose (experiment 2). Brains were removed, tissue containing the hypothalamus and POA dissected out, infiltrated with 30% sucrose, and frozen coronal sections (50 or 40 μm thick for experiments 1 and 2, respectively) cut and stored at −20 C in cryoprotectant.

ICC

Antibodies. Guinea pig antisera against NKB (37) and rabbit antisera against kisspeptin (27) were provided by P.C. and A.C., respectively. Rabbit antiserum against dynorphin A (33, 34) was purchased from Phoenix Pharmaceuticals, Inc. (Burlingame, CA), and guinea pig antisera against mouse AGRP (82–131) and γ-MSH were obtained from Antibodies Australia (Melbourne, Australia). Preabsorption of the latter two antisera with 0.5 mg/ml of the original peptide abolished all staining in the ovine ARC (data not shown).

Because kisspeptin is a member of a large family of RF-amide peptides (38), questions have been raised about the specificity of cells identified using ICC (2, 19). Therefore, we first analyzed the immunocytochemical characteristics of the rabbit polyclonal antibody against mouse kisspeptin-10 provided by A.C. (27), comparing it with a commercially available (Phoenix Pharmaceuticals, Inc.) rabbit polyclonal antibody against human kisspeptin-10. To determine whether kisspeptin antibody cross-reacts with other RF-amide peptides, we preincubated antiserum with kisspeptin (Metastin 45-54-amide, human), GnRH (Auspep, Parkville, Melbourne, Australia), quail gonadotropin-inhibiting hormone (GnIH), human neuropeptide FF (NFF), Chemerin (145–157-amide, human), pyroglutamylated-RF-amide peptide (QRFP-43; human 154–196-amide), and bovine prolactin-releasing peptide (PrRP) (a gift from Dr. S. Anderson, The University of Queensland, Brisbane, Australia). Kisspeptin, GnIH, NFF, Chemerin, and QRFP-43 were all purchased from Phoenix Pharmaceuticals, Inc. Antiserum (diluted 1:2000) was preabsorbed with three concentrations of RF-amide (100, 10, and 1 μg/ml) in a Tris-buffered saline (TBS) cocktail containing 10% normal goat serum (NGS) and 0.3% Triton X-100 (NGS) overnight at 4 C. The antibody-peptide mixture was then assessed using a previously described ICC procedure (36). Briefly, sections through the middle ARC of luteal-phase ewes were mounted onto slides and left overnight to dry. Antigen retrieval was performed with 1 M citrate buffer (pH 6) in a microwave oven at 1000 W (2 × 5 min). Sections were washed in TBS and incubated with blocking solution (NGS) at room temperature (RT). The antibody-peptide mixture was then applied, and slides were incubated for 72 h at 4 C. Slides were then washed in TBS and sections incubated with goat antirabbit conjugated to Alexa 488 (1:500; Molecular Probes, Inc., Eugene, OR) for 1 h at RT. Sections were again washed in TBS and then counterstained with 0.3% Sudan black B. After further washes, coverslips were applied with fluorescence mounting medium (Dako, Carpinteria, CA). A positive control (no antibody preabsorption) and a negative control (antibody preabsorbed with kisspeptin) were included in every procedure.

Experiment 1: colocalization of kisspeptin and dynorphin. To visualize both kisspeptin and dynorphin using antibodies raised in rabbits, we used tyramide amplification solution (TSA) as previously described (33, 39) using free-floating sections. Briefly, after washing in 0.1 M PBS, sections were incubated in PBS containing 0.4% Triton X-100 and 20% NGS for 1 h at RT, and then for 17 h at RT with 1:300,000 anti-kisspeptin antiserum. The primary antisera were then labeled (1 h at RT) with biotinylated donkey antirabbit sera (1:500 in NGS; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), and sections were sequentially incubated for 1 h in ABC-elite (1:500 in PBS; Vector Laboratories, Burlingame, CA) for 10 min in TSA (1:250 in PBS; New England Nuclear Life Science Products Life Sciences, Boston, MA), and for 30 min in streptavidin-Alexa 488 (1:100 in PBS; Molecular Probes) with washings in between each incubation. After thorough washing in PBS, sections were incubated with dynorphin antisera (1:1000 in NGS) for 17 h, washed, and incubated in Alexa 555 conjugated to donkey antirabbit (1:100 in NGS; Molecular Probes) for 30 min. Finally, sections were washed, mounted on glass slides, dried, and coverslipped with gelvatol (33). Controls included omission of either primary antibody and resulted in a complete lack of staining for the corresponding peptide.

Numbers of neurons immunopositive for dynorphin, kisspeptin, or both, were counted in sections containing rostral, middle, or caudal levels of the ARC (four sections each), or POA (six to eight sections each) at ×20 magnification using a Nikon Microphot (Tokyo, Japan). For each ewe and for each brain region, the degree of double labeling was calculated both as the percentages of the total number of kisspeptin-ir cells containing dynorphin and the percentages of the dynorphin-positive cells containing kisspeptin.

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Experiment 2: colocalization of kisspeptin and NKB, AGRP, or γ-MSH. Because hypothalamic NKB-containing perikarya are limited to the ARC (33, 35) in the sheep, we only examined this region in experiment 2; TSA amplification was not used because primary antisera were from different species. Sections from the rostral, middle, or caudal ARC were washed in 0.05 M PBS, mounted on slides, and dried overnight. Kisspeptin-containing cells were labeled using antigen retrieval and kisspeptin antibody (1:2000), as described above in *Materials* and *Methods*, except that Alexa 546 (Molecular Probes) was used in place of Alexa 488 as fluor. After incubation of slides with 1:500 goat antirabbit sera conjugated to Alexa 546, they were washed three times with PBS and then incubated in blocking sera (10% NGS and 0.3% Triton X-100 in PBS). Sections were next exposed to guinea pig antisera against NKB (1:1000), AGRP (1:1000), or MSH (1:500) for 48 h at 4 C. After washing, sections were incubated in 1:250 goat antiguinea pig conjugated to Alexa 488 (Molecular Probes) for 1 h at RT, washed in PBS, and coverslipped with fluorescence mounting medium. Controls included omission of either primary antisera, which eliminated the fluorescent signal of the appropriate antigen without affecting staining for the other neuropeptide.

Separate images of immunoreactive cell bodies were captured with the appropriate excitation for Alexa 488 and Alexa 546, and computer software used to superimpose the two images. A single observer then counted the total number of immunopositive cell bodies, and the number of cells containing both kisspeptin and NKB, MSH, or AGRP in the rostral, middle, and caudal ARC (one section per region per ewe). For each ewe, the degree of double labeling was calculated as a percentage of the total number of kisspeptin-ir cells and the percentage of NKB, AGRP, or MSH neurons also containing kisspeptin.

Statistical analysis

The number of each cell type per section within an area was compared by one-way ANOVA with repeated measures (experiment 1) or two-way ANOVA with repeated measures (experiment 2, main effects stage of cycle and anatomical area). The percentage colocalization was transformed using arcsine of the square root before analysis by one or two-way ANOVA with repeated measures. Statistical significance was $P < 0.05$.

Results

Antibody absorption tests

Representative photomicrographs of kisspeptin immunostaining in the ovine ARC after antibody preabsorption experiments are shown in Fig. 1. The ability of kisspeptin antiserum (against both human and mouse sequence) to identify cells in the ovine ARC using ICC was diminished by preabsorption of the antiserum with 1–100 μg/ml kisspeptin. Faintly stained cells were still observed with the antimouse kisspeptin antiserum after preabsorption, consistent with previous reports (27). Preabsorption of the antihuman-kisspeptin antiserum with GnIH or NFF peptide (concentration 1–100 μg/ml) completely blocked immunostaining in the ARC. The same peptide preabsorption of the antimouse-kisspeptin antiserum had no effect, with kisspeptin immunoreactive cells and fibers remaining detectable. Preabsorption of either kisspeptin antiserum with GnRH, Chemerin, QRFP-43, or PrRP (1–100 μg/ml) had no effect on kisspeptin immunoreactivity. These data indicate that the commercially available antisera against human kisspeptin is not as specific in our hands as the antibody against mouse kisspeptin, which could explain differences in the distribution of kisspeptin-positive neurons observed with these two antibodies in sheep (27, 36). Therefore, all subsequent work was done with the antiserum against mouse kisspeptin.

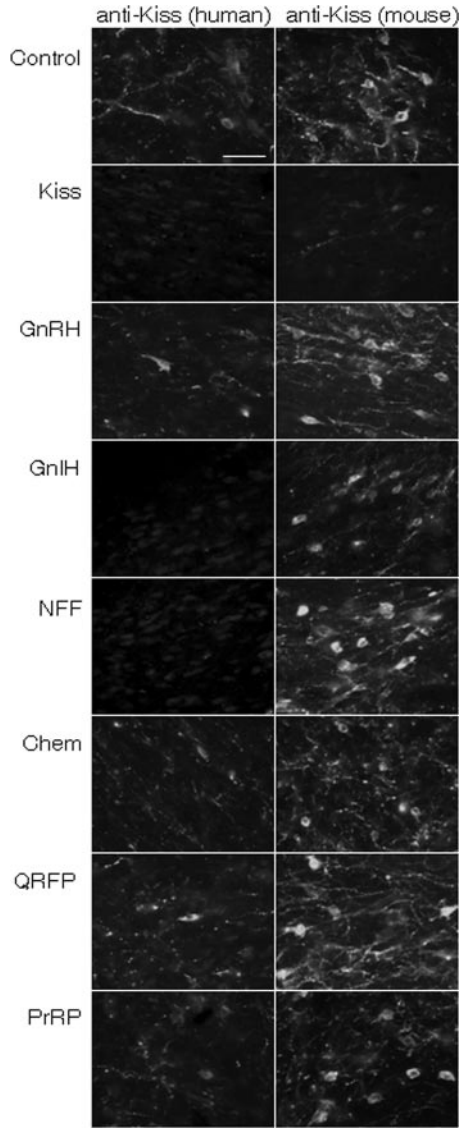

FIG. 1. Controls for specificity of the antisera to the human (*left column*) or to the murine (*right column*) forms of kisspeptin (see *Materials and Methods*). Images of the ARC after antibody preabsorption with kisspeptin (Kiss), GnRH, GnIH, NFF, Chemerin (Chem), pyroglutamylated-RF-amide peptide (QRFP), or PrRP, at a concentration of 1 μg/ml. Control is no preabsorption of antibody. Note virtual abolition of labeling by preabsorption of the antihuman kisspeptin by GnIH and NFF. All photomicrographs were taken using a ×40 objective. *Scale bar* representative for all images, 50 μm.

Experiment 1: colocalization of kisspeptin and dynorphin A

The distribution of dynorphin-containing cells was similar to that reported previously (34, 40), with immunopositive cells observed in the magnocellular neurons of the paraventricular and supraoptic nuclei (data not shown), in the periventricular region of the POA and anterior hypothalamic area, and throughout the ARC, with a concentration in the middle and caudal portions of this nucleus (Fig. 2). Immunopositive kisspeptin neurons were also found primarily in the ARC, with more kisspeptin-positive neurons per section in the middle and caudal ARC than the rostral portion of this

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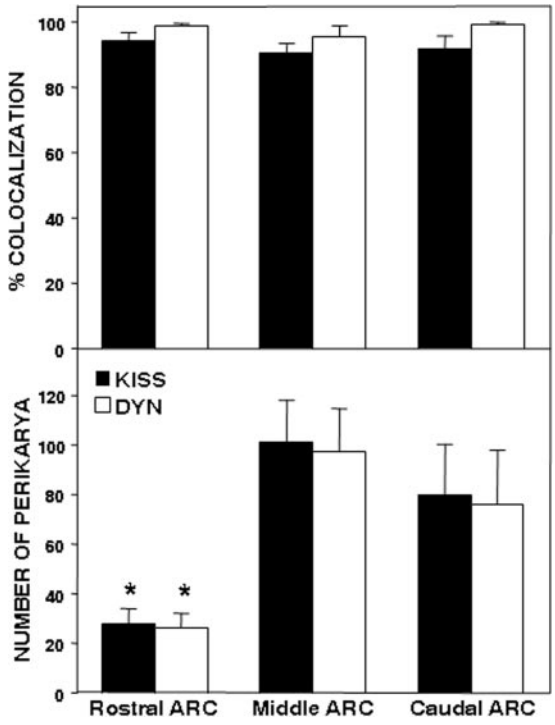

FIG. 2. Colocalization of kisspeptin (KISS) and dynorphin (DYN) in the ARC. *Bottom*, Mean (±SEM) number of perikarya per slide containing either kisspeptin (*solid bars*) or dynorphin (*open bars*) in the rostral, middle, and caudal regions of the ARC. *Top*, Mean (±SEM) percentage of kisspeptin perikarya containing dynorphin (*solid bars*) and dynorphin perikarya containing kisspeptin (*open bars*) in the rostral, middle, and caudal regions of the ARC. *, $P < 0.5$ compared with the number of perikarya in middle and caudal regions.

nucleus. Kisspeptin-immunoreactive neurons were also observed in the medial POA.

Extensive colocalization of dynorphin and kisspeptin immunostaining was evident in the ARC (Fig. 3). Of the total number of ARC kisspeptin neurons examined (459 ± 62 per ewe), over 94% also contained dynorphin. Similarly, 95% of ARC dynorphin-containing cell bodies (440 ± 70 per ewe) were also positive for kisspeptin. This degree of colocalization was substantially greater than we observed in a preliminary report (41), probably because that work was done with the less-specific commercially available antibody to kisspeptin. There were no differences in the degree of colocalization among the rostral, middle, and caudal regions of the ARC (Fig. 2, *top panel*). Extensive colocalization of dynorphin and kisspeptin was also evident in dendrites, fibers, and varicosities within the ARC (Fig. 3). In contrast, no colocalization of these two neuropeptides was observed within cell bodies (264 ± 22 kisspeptin-positive per ewe; 38 ± 4 dynorphin-positive per ewe) in the POA, although fibers containing both kisspeptin and dynorphin were seen in this area (Fig. 4). In addition, we noted numerous instances of close contacts between double-labeled fibers and kisspeptin cells in the ARC (Fig. 3), as well as in the POA (Fig. 4).

Experiment 2: colocalization of kisspeptin and NKB, AGRP, or γ-MSH

No effects of stage of cycle were observed on either cell number or levels of coexpression (data not shown), so data from all three stages of the cycle were combined (n = 9). The distribution of kisspeptin-immunopositive cells within the ARC was similar to that observed in experiment 1, with fewer cells per section in the rostral, than in the middle and caudal, regions of this nucleus (Fig. 5). As expected (35), there was also fewer NKB-containing cells per section in the rostral ARC than in the other two regions. In contrast, there were no significant differences among regions within the ARC in either γ-MSH- or AGRP-immunopositive cell bodies (Fig. 6).

As was the case with dynorphin, extensive colocalization of kisspeptin and NKB was observed (Fig. 7). More than three quarters (80.4 ± 3.8%; n = 9) of the 175 ± 33 kisspeptin neurons observed per ewe also contained NKB. Conversely, 72.9 ± 4.7% of the 161 ± 26 NKB-positive cell bodes per ewe also contained kisspeptin. There was a tendency for a lower level of coexpression in the rostral ARC (Fig. 5), but this was not statistically significant. In contrast, no colocalization of kisspeptin and γ-MSH or kisspeptin and AGRP was ob-

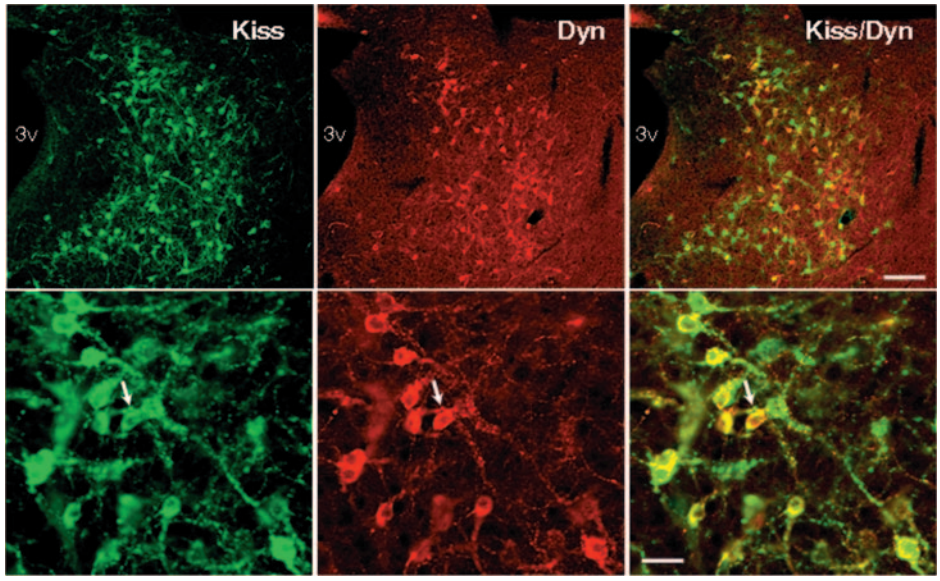

FIG. 3. Confocal microscopic images of a section through the caudal ARC stained for both kisspeptin (Kiss) (*green fluorescence*) and dynorphin (Dyn) (*red fluorescence*) at low (*top*) and high (*bottom*) magnifications. *Panels on the right* are computer-generated overlays of *left and middle panels* illustrating colocalization of kisspeptin and dynorphin (*yellow fluorescence*). *Arrows in the bottom panel* indicate an example of a double-labeled kisspeptin/dynorphin fiber in close contact with a kisspeptin/dynorphin cell body. *Bar in top right panel*, 100 μm. *Bar in bottom right panel*, 20 μm.

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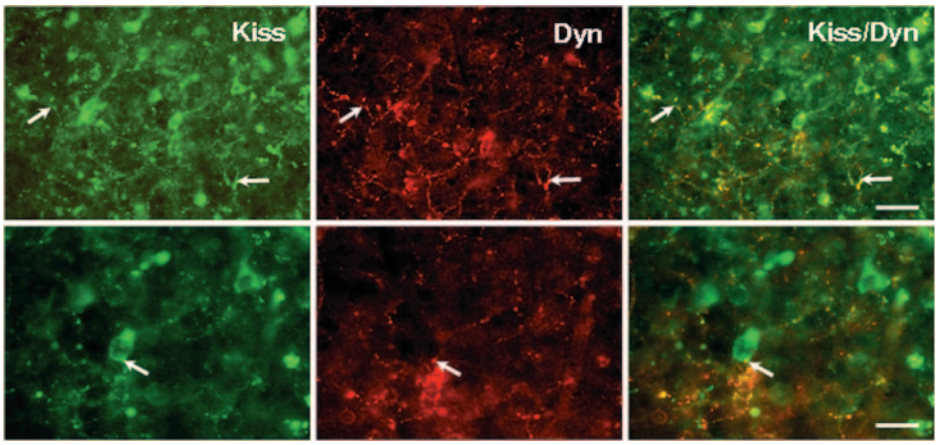

FIG. 4. Lack of colocalization of kisspeptin (Kiss) and dynorphin (Dyn) in POA. *Top and bottom panels* are medium and high-magnification images, respectively, of a section stained for both kisspeptin (*green fluorescence*) and dynorphin (*red fluorescence*). As in Fig. 3, the *right panels* are overlays showing lack of dynorphin colocalization in POA kisspeptin perikarya, although several examples of double-labeled fibers (*arrows*) can be seen. *Arrows in the bottom panels* point out an example of a double-labeled varicosity in close contact with a single-labeled kisspeptin cell body. *Bar in top right panel*, 50 μm. *Bar in bottom right panel*, 20 μm.

served (Fig. 7). None of the 1878 γ-MSH-positive and 808 AGRP-positive cell bodies costained for kisspeptin; over 1000 kisspeptin cell bodies were examined for colocalization of each of these peptide, and no dual staining was observed.

Discussion

We present conclusive evidence to show that, in the ARC of the ewe, essentially all kisspeptin neurons express dynorphin, and most also express NKB. In contrast, these cells do not produce γ-MSH or AGRP. Although recent indirect evidence suggested that kisspeptin neurons also contain NKB in women (28), this is the first direct evidence that kisspeptin neurons contain other neuropeptides implicated in the control of GnRH secretion. Given the high degree of colocalization of dynorphin and NKB previously reported in the ewe (33), and the high percentage of dynorphin and NKB neurons that also contain kisspeptin, we propose that a single subpopulation of neurons in the ARC contains all three neuropeptides. Moreover, this subpopulation appears to be distinct from the orexigenic (producing AGRP and neu-

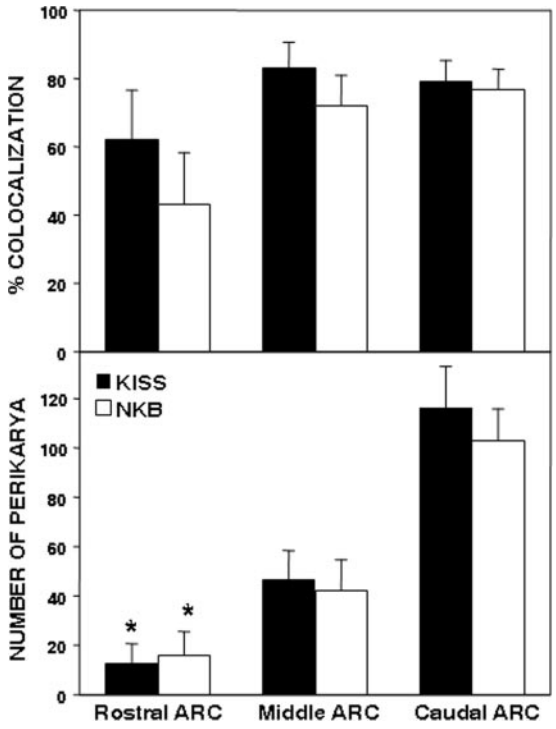

FIG. 5. Colocalization of kisspeptin (KISS) and NKB in the ARC. *Bottom*, Mean (±SEM) number of perikarya per slide containing either kisspeptin (*solid bars*) or NKB (*open bars*) in the rostral, middle, and caudal regions of the ARC. *Top*, Mean (±SEM) percentage of kisspeptin perikarya containing NKB (*solid bars*) and NKB perikarya containing kisspeptin (*open bars*) in the rostral, middle, and caudal regions of the ARC. *, $P < 0.5$ compared with the number of perikarya in middle and caudal regions.

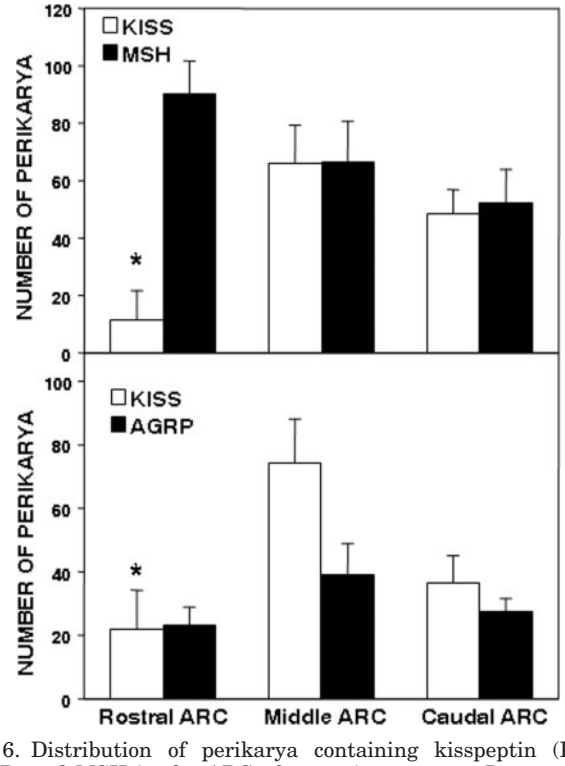

FIG. 6. Distribution of perikarya containing kisspeptin (KISS), AGRP, and MSH in the ARC of ovary intact ewes. *Bottom*, Mean (±SEM) number of perikarya per slide containing either kisspeptin (*open bars*) or AGRP (*solid bars*) in the rostral, middle, and caudal regions of the ARC. *Top*, Mean (±SEM) number of perikarya per slide containing either kisspeptin (*open bars*) or γ-MSH (*solid bars*) in the rostral, middle, and caudal regions of the ARC. No colocalization of kisspeptin and either AGRP or MSH was observed. *, $P < 0.5$ compared with the number of perikarya in the middle region.

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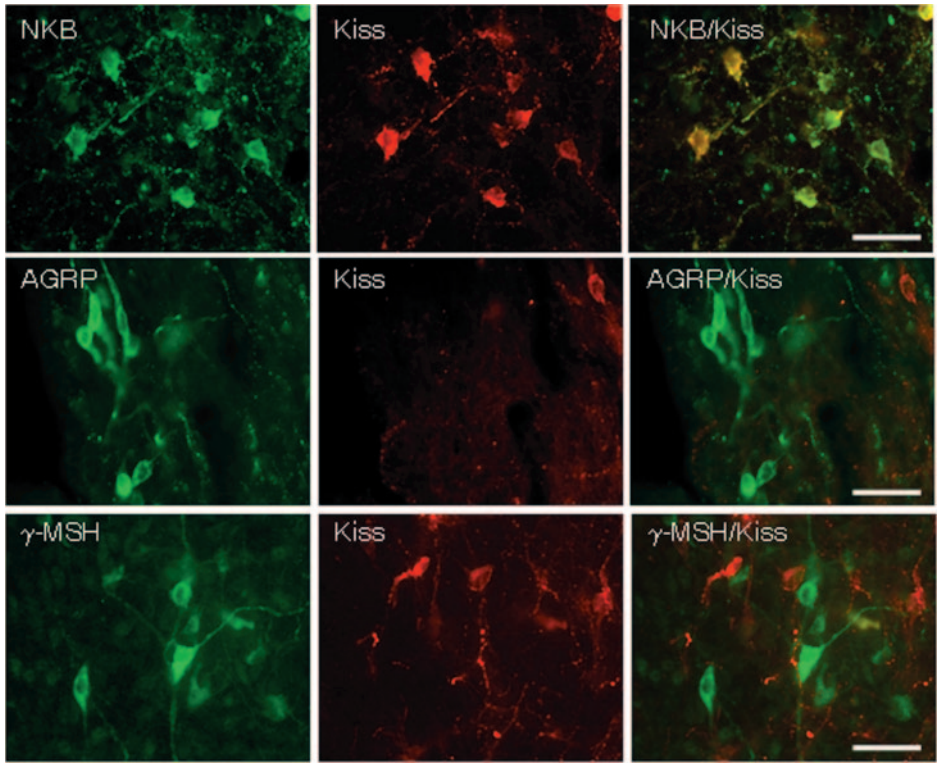

FIG. 7. Fluorescence images of the middle ARC immunostained for kisspeptin (Kiss) and NKB (*top*), kisspeptin and AGRP (*middle*), and kisspeptin and γ-MSH (*bottom*). In all cases, kisspeptin neurons are identified by *red fluorescence* (*middle*), whereas the second neuropeptide is identified by *green fluorescence* (*left*). *Panels on the right* are overlays of red and green fluorescent images, illustrating colocalization of kisspeptin and NKB but the absence of kisspeptin-AGRP and kisspeptin-MSH colocalization.

ropeptide Y) and anorexigenic (producing melanocortins and β-endorphin) neurons in this nucleus.

These data are consistent with previous reports that over 90% of dynorphin (34), NKB (35), and kisspeptin (17, 27) neurons in the ovine ARC contain ERα and/or PR. In contrast, only a small percentage (15–20%) of neuropeptide Y (42), β-endorphin (43), and dopaminergic (42, 43) neurons in the ovine ARC express ERα. Approximately half the ARC glutamatergic neurons contain ERα (44) in ewes, suggesting that some of them may overlap with the kisspeptin/dynorphin/NKB subpopulation. Gonadal steroid receptors have also been found in a high percentage of rodent NKB (45), dynorphin (46), and kisspeptin neurons (21, 22), and most likely human NKB neurons (47, 48), whereas much lower percentages of other neuropeptide cell populations contain receptors for these steroids in rats (49–51), guinea pigs (52–54), and primates (55). Because virtually all of these neurons express ERα and PR, they represent a population that likely relays the feedback effects of these steroids to GnRH neurons to regulate reproductive function. It remains to be determined how a single cell type can function to exert both negative and positive feedback effects, but the presence of these three neuropeptides raises some interesting possibilities discussed below.

These data, together with earlier anatomical studies, provide strong indirect evidence that ARC kisspeptin neurons project directly to GnRH cell bodies in the ewe. Specifically, previous work in sheep has demonstrated synaptic input to GnRH neurons that contain NKB (35) or dynorphin (56), and more recently we have observed colocalization of dynorphin and NKB in neuronal afferents in close contact with GnRH neurons (57), indicating that these inputs occur from ARC neurons containing both neuropeptides (33). Kisspeptin-containing afferents have been shown in close association with GnRH neurons in the rat (23), but whether these occur from AVPV or ARC kisspeptin cells remains to be determined. Two other important characteristics of kisspeptin neurons may also be inferred from these data showing the presence of all three peptides in the same ARC cells. Kisspeptin may well be involved in progesterone negative feedback, and its expression is likely to be sexually dimorphic in this species. The former inference is derived from the colocalization of kisspeptin and dynorphin because several lines of evidence suggest that these dynorphin-containing neurons mediate progesterone negative feedback. First, as noted previously, almost all of these cells contain PR (34). Second, dynorphin synapses are evident on many ovine GnRH neurons, and an antagonist to the dynorphin (κ-opioid) receptor stimulates episodic LH secretion in luteal phase ewes (56). Finally, progesterone increases dynorphin levels in cerebrospinal fluid collected from the third ventricle of ovariectomized ewes and expression of mRNA levels for preprodynorphin (58).

The colocalization of kisspeptin and NKB raises the possibility that the ARC kisspeptin neurons are sexually dimorphic because ewes have approximately twice as many NKB neurons in the ARC than their male counterparts. We have obtained preliminary data that this sexual dimorphism also occurs in the number of ARC neurons expressing both dynorphin (59) and kisspeptin (60). Because E_2 can induce an LH surge in ewes, but not rams (61, 62), the sexual dimorphism of ARC kisspeptin expression suggests that these neurons may mediate the positive feedback actions of E_2, as do the sexually dimorphic AVPV kisspeptin neurons in the rat (21–26). However, the negative feedback actions of progesterone (63) and E_2 (61, 64) are also sexually dimorphic in sheep, so a role in these feedback actions of steroids cannot be ex-

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cluded. Because ewes are more sensitive to the negative feedback actions of these steroids than rams, the higher expression of the inhibitory neurotransmitter, dynorphin, in ewes may be more important for these sex differences than the increase in stimulatory peptides, like kisspeptin.

The ARC population of kisspeptin neurons appears to be conserved throughout several mammalian species since they have now been identified in mice (21, 22), rats (6, 20), hamsters (18, 19), sheep (17, 27), monkeys (14, 28), and humans (28). Moreover, in all these species, kisspeptin expression in the ARC appears to be inhibited by ovarian steroids, suggesting a functional conservation as well. Whether the population of kisspeptin-dynorphin-NKB containing neurons found in sheep is also conserved awaits work in other species, but two studies provide some support for this possibility. First, there is direct evidence in the rat for extensive colocalization of NKB and dynorphin in the ARC; because all of these neurons contain ERα (46), it is very likely that they also produce kisspeptin. Second, the similar distribution and morphology of NKB- and kisspeptin-containing neurons in the infundibular (ARC) nucleus of postmenopausal women led Rometo *et al*. (28) to propose that these two neurotransmitters are contained in the same subpopulation of cells. Interestingly, the dynorphin-NKB containing subpopulation of the ARC appears to possess extensive reciprocal connections among its individual constituent neurons, with dynorphin-NKB fibers occurring locally synapsing on dynorphin-NKB cell bodies and dendrites in both sheep (33) and rats (46). Our observations that kisspeptin/dynorphin fibers also form close contacts onto kisspeptin cells of the ARC is consistent with these reciprocal connections seen previously, and suggests that the effects of gonadal steroids or other signals upon individual kisspeptin neurons may be rapidly communicated to other cells in this subpopulation, as well as to kisspeptin cells in the POA (Fig. 4).

It is well established that dynorphin inhibits and kisspeptin stimulates GnRH release, whereas both inhibitory (65) and stimulatory (66) effects of an agonist to the NKB receptor have been observed in rats and sheep, respectively. The presence of these three neuropeptides in the same neurons suggests that ovarian steroids are likely to have differential effects on their expression. Indeed, ovariectomy increased kisspeptin expression in the ARC of several species (17, 21, 22, 28), whereas it reduced the level of mRNA for prepro-dynorphin in the ewe (58). Stimulatory effects of ovariectomy and inhibitory effects of E_2 on NKB gene expression have been reported in rats (67), sheep (68), monkeys (69), and humans (47), suggesting a stimulatory role for this neuropeptide. However, differential effects of ovarian steroids on expression of these three neurotransmitters have yet to be examined in the same animals. It is also tempting to speculate that differential effects on expression of κ and GPR54 receptors in GnRH neurons could provide a simple mechanism for switching from the negative to positive feedback effects of E_2. At this time there is insufficient information on expression of these receptors in GnRH neurons to assess this hypothesis.

In summary, these data demonstrate that ARC kisspeptin neurons in the ewe express both dynorphin and NKB. Thus, we propose that a single population of ARC neurons contains all three neuropeptides. These observations raise intriguing questions about the physiological roles of these neurons and their differential regulation by ovarian steroids.

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