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KiSS-1 Messenger Ribonucleic Acid Expression in the Hypothalamus of the Ewe Is Regulated by Sex Steroids and Season

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The KiSS-1 gene encodes a family of peptides called kisspeptins, which are endogenous ligands for the G protein-coupled receptor GPR54. Kisspeptin function appears to be critical for GnRH secretion and the initiation of puberty. To test the hypothesis that steroid hormones regulate KiSS-1 mRNA expression in the ewe, we examined the brains of ovary-intact (luteal phase) and ovariectomized (OVX) ewes, as well as OVX ewes that received estradiol (E) or progesterone (P) replacement. KiSS-1 mRNA-expressing cells were predominantly located in the arcuate nucleus (ARC). Here, expression was increased after OVX but returned to the level of gonad-intact animals with E treatment. Treatment with P partially restored KiSS-1 expression toward gonad-intact levels. Double-label immunohistochemistry revealed that approximately 86% of kisspeptin-immunoreactive cells in the ARC are also P-receptor positive. Finally, we tested the hypothesis that KiSS-1 mRNA is lower during anestrus, due to a non-steroid-dependent seasonal effect. In OVX ewes, expression in the ARC was lower at the time of year corresponding to anestrus. We conclude that KiSS-1 expression in the ARC of the ewe brain is negatively regulated by chronic levels of E and P, suggesting that both steroids may exert negative feedback control on GnRH secretion through altered kisspeptin signaling. Furthermore, a seasonal alteration in KiSS-1 expression in the ARC of OVX ewes strongly suggests that kisspeptin is fundamentally involved in the control of seasonal changes in reproductive function. (*Endocrinology* 148: 1150–1157, 2007)

KISSPEPTINS ARE A family of neuropeptides derived from the translation product of the KiSS-1 gene (1–4). Posttranslational processing of a 145-amino-acid precursor peptide results in the formation of smaller C-terminal peptides (kisspeptin-54, -14, -13, and -10), which activate the G protein-coupled receptor GPR54 with equal efficacy (1–4). In humans and mice, inactivating mutations in GPR54 result in the failure to initiate puberty and subsequent hypogonadotropic hypogonadism (5–7). Practically all GnRH neurons express GPR54 (8–10), and central and peripheral administration of kisspeptin stimulates GnRH and gonadotropin secretion in various species (11–14). Kisspeptin-producing neurons (or KiSS-1 neurons) have been localized to various regions of the forebrain in rodents, primates, and sheep (11, 14–17). In sheep, most KiSS-1 neurons are found in the arcuate nucleus (ARC), with a smaller population present in the preoptic area (POA) (15, 16, 18).

In the female, the pulsatile release of GnRH is tightly regulated by the negative and positive feedback of gonadal steroids (19–21). Although GnRH neurons express estrogen receptor (ER)β (22), they do not express ERα (23, 24) or progesterone receptor (PR) (25), and so other steroid-sensitive neurons must mediate the feedback effects of sex steroids on GnRH secretion. In female rodents, ovarian steroids inhibit KiSS-1 mRNA expression in the ARC but stimulate expression in the anteroventral periventricular nucleus (AVPV) (26, 27). These observations have led to the notion that kisspeptin-producing neurons in the former mediate the negative-feedback effects of estrogen (E), whereas the positive-feedback effects of steroids on GnRH cells are mediated by kisspeptin-producing neurons in the latter (26, 27). This is consistent with our understanding that the AVPV is the surge center in the rodent (28, 29), but this is not the case in the sheep. Indeed, implantation of E into the mediobasal hypothalamus elicits GnRH/LH surges in the ewe (30). Consistent with the notion that the GnRH/LH surge center is within the basal hypothalamus and that kisspeptin may relay the positive-feedback effect of E to GnRH cells, we recently reported that KiSS-1 mRNA is up-regulated in the ARC, just before and during the GnRH/LH surge in the ewe (15). Unlike the female rat, the ewe does not show up-regulation of KiSS-1 in the POA of the brain at the time of the cyclic surge. Thus, it appears that both negative- and positive-feedback effects of E and progesterone (P) on GnRH secretion in the sheep may be mediated by KiSS-1 neurons in the ARC. Whether E or P is able to inhibit KiSS-1 expression in the ARC is unknown, although we have shown that kisspeptin immunoreactivity is increased by ovariectomy (18).

In sheep, reproductive activity is seasonal, being activated by short-day photoperiod and inhibited by long days. Dur-

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Abbreviations: ARC, Arcuate nucleus; AVPV, anteroventral periventricular nucleus; E, estrogen; ER, estrogen receptor; ir, immunoreactive; P, progesterone, POA, preoptic area; PR, progesterone receptor; TBS, Tris-buffered saline.

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ing the nonbreeding (anestrous) season, GnRH secretion is reduced due to steroid-independent (31–33) and steroid-dependent (34, 35) effects of photoperiod. We hypothesize that the mechanism underlying the regulation of GnRH secretion and, in turn, seasonal changes in reproductive function is changing KiSS-1 mRNA expression.

The primary objectives of the present study were 3-fold. The first was to determine the regulation of KiSS-1 mRNA by E or P. Accordingly, we used single-label *in situ* hybridization to compare expression of KiSS-1 mRNA in groups of ovary-intact (luteal phase) and ovariectomized (OVX) ewes as well as OVX ewes receiving chronic E or P replacement. The second objective was to test whether P is likely to act directly on kisspeptin neurons, using double-label immunohistochemistry for kisspeptin and PR. Our final objective was to test the hypothesis that KiSS-1 mRNA is regulated across the breeding and nonbreeding seasons of the ewe in a steroid-independent manner. Accordingly, we examined expression of KiSS-1 in the brains of OVX ewes killed in February (early breeding season), May (late breeding season), October (mid nonbreeding season), and December (late nonbreeding season).

Materials and Methods

Animals

Corriedale ewes of similar age (5–6 yr) and weight were maintained under natural conditions at the Monash University Sheep Facility, Werribee, Victoria. All experiments were carried out according to the guidelines established by the Australian Prevention of Cruelty to Animals Act 1986 and was approved by the Monash University Animal Ethics Committee. Brains of ovary-intact animals were collected during the luteal phase of the estrous cycle. The estrous cycles of ovary-intact animals were synchronized by im injection of 125 μg of the synthetic luteolysin Cloprostenol (Estrumate; Pitman-Moore, Sydney, Australia), and brains were collected on d 10 of the ensuing estrous cycle (luteal phase). Verification of the stage of cycle for these animals has been previously published (36). Briefly, ovaries contained at least one active corpus luteum, plasma LH concentrations were less than 1.0 ng/ml, and plasma P concentrations were elevated (3.2 $\pm$ 0.5 ng/ml).

Ovariectomy and steroid replacement

Ovariectomy was performed as previously described (37). Chronic E treatment was achieved with a 3-cm sc implant [SILASTIC brand tubing (Dow Corning Corp., Midland, MI) with an inner diameter of 3.35 mm and outer diameter of 4.65 mm packed with crystalline estradiol-17β (Sigma Chemical Co., St. Louis, MO). Implants were inserted into the axillary region 2 wk before tissue collection. These implants were designed to produce circulated E levels of approximately 3–5 pg/ml (38), similar to that observed in the normal luteal phase. P treatment was via intravaginal controlled internal drug release devices containing 0.3 g P (Riverina; Artificial Breeders Ltd., Albury, Australia). Controlled internal drug release devices were inserted 2 wk before tissue collection, and plasma levels of P in these animals, which were previously described (39), were 2.0 $\pm$ 0.3 ng/ml.

Experimental design

Experiment 1. The purpose of this experiment was to determine the effects of ovariectomy and E or P replacement on hypothalamic KiSS-1 mRNA. Ewes were divided into the following four groups: ovary intact (luteal, n = 6), OVX (n = 5), OVX plus E replacement (n = 4), and OVX plus P replacement (n = 4). The animals were treated during the normal breeding season, and ovariectomy took place 5–7 wk before tissue collection. Hypothalami were collected for KiSS-1 mRNA *in situ* hybridization as follows. The ewes were killed by an iv overdose of sodium pentobarbital (Lethabarb; Virbac, Peakhurst, Australia), and the heads were perfused with 2 liters of heparinized saline (12.5 U/ml) followed by 2 liters of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) and 1 liter of the same fixative containing 20% sucrose. The brains were removed and the hypothalamus dissected out and postfixed in fixative containing 30% sucrose for 7 d and then frozen in powdered dry ice. Coronal sections (20 μm) were cut on a cryostat (from the diagonal band of Broca to the mammillary bodies) and placed into cryoprotectant (30% ethylene glycol, 20% glycerol in sodium phosphate buffer) with 2% paraformaldehyde and stored at −20 C until used for *in situ* hybridization.

Experiment 2. The purpose of this experiment was to determine whether PR is present in KiSS-1 neurons in the ARC of the ewe brain. Brains were collected for double-label immunohistochemistry for kisspeptin and PR from three ovary-intact animals (luteal phase) as described above with the following modifications. These brains were perfused with 2 liters of heparinized saline followed by 2 liters of fixative, containing 4% paraformaldehyde plus 15% picric acid in 0.1 M phosphate buffer (pH 7.4) and 1 liter of the same fixative containing 20% sucrose. The hypothalamus was dissected out and then placed in a solution of 0.1 M phosphate buffer containing 30% sucrose. Coronal sections (40 μm) were placed in cryoprotectant without paraformaldehyde and stored at −20 C until used for immunohistochemistry.

Experiment 3. The purpose of this experiment was to determine whether KiSS-1 mRNA expression in the hypothalamus changes in a steroid-independent manner between the normal breeding and nonbreeding seasons. Sheep are short-day breeders, and for this breed in this location, the normal breeding season (when ewes exhibit regular estrous cycles) is between February and June, with transitional periods (with irregular cycles or silent estrus) in January and July (Clarke, I. J., and B. D. Doughton, unpublished data). In this experiment, animals were run on pasture, so that they were subject to the normal change in photoperiod as well as environmental fluctuations in temperature. Groups of OVX ewes were sampled in February (early breeding season, n = 4), May (mid-breeding season, n = 4), October (mid nonbreeding season, n = 6), and December (late nonbreeding season, n = 5). OVX ewes were used so that we could discern the effect of season in the absence of fluctuating levels of gonadal steroids, because the feedback effects of E and P change with season (34), and previous experiments showed an effect of these steroids on KiSS-1 mRNA (17, 26). Collection of the brains and processing of the hypothalami were as in experiment 1, and *in situ* hybridization was performed to quantify KiSS-1 expression.

Radiolabeled KiSS-1 cRNA riboprobe

A 375-base sequence of the ovine KiSS-1 gene (GenBank accession no. DQ059506) was inserted into a pGemT-easy plasmid. The antisense ovine KiSS-1 probe was transcribed from linearized plasmid containing the ovine KiSS-1 insert with SP6 polymerase (Promega Corp., Madison, WI) and [^{35}S]UTP (GE Healthcare Life Sciences, Boston, MA) under a standard transcription protocol (40). The riboprobe was separated from unincorporated nucleotides on a Sephadex G-25 column.

In situ hybridization

In situ hybridization was performed as described for KiSS-1 expression in ewes during the estrous cycle (15). Three sections representing the rostral, medial, and caudal regions of the ARC and three sections through the POA were chosen from each ewe, mounted on SuperFrost slides, and prepared for *in situ* hybridization. Radiolabeled (^{35}S) antisense KiSS-1 riboprobe was denatured, diluted in hybridization buffer at a concentration of 5×10^6 cpm/ml along with tRNA, and applied to slides (120 μl/slide). After hybridization (53 C for 16 h), slides were treated with RNase A, washed in decreasing concentrations of saline-sodium citrate, and dehydrated. Slides were then dipped in Ilford K5 photographic emulsion (Ilford Imaging, Melbourne, Australia), stored in the dark at 4 C, and developed 7–10 d later. No signal was observed after the application of radiolabeled sense probe (data not shown).

KiSS-1 quantification and analysis

Image analysis was carried out using randomly coded slides under dark-field illumination with custom-designed software designed to

count the total number of cells and the number of silver grains per cell (41). Cells were counted when silver grain density was more than five times background. Data are expressed as the mean number of identifiable KiSS-1 cells and the mean number of silver grains per cell (a semiquantitative index of mRNA expression per cell).

Double-label immunohistochemistry for kisspeptin and PR

Three sections representing the rostral, medial, and caudal regions of the ARC were chosen from each ewe and mounted on SuperFrost slides. Antigen retrieval was performed using 1 M citrate buffer (pH 6) in a microwave oven at 1000 W (2 × 5 min). After cooling (20 min), the sections were washed in Tris-buffered saline (TBS), and a blocking solution containing 10% normal goat serum and 0.3% Triton X-100 in TBS was applied. Sections were then incubated for 72 h at 4 C with a cocktail containing a rabbit polyclonal antibody against mouse kisspeptin-10 (no. 566 used at a dilution of 1:2000) (16) and a mouse monoclonal antibody against the PR (Ab-8 used at a dilution of 1:100; Neomarkers, Union City, CA). Both antibodies have been used successfully and their specificity tested previously (16, 25). Slides were then washed in TBS and sections incubated with a mixture of goat antirabbit Alexa 448 and goat antimouse Alexa 546 for 2 h at room temperature (both 1:400; Molecular Probes Inc., Eugene, OR). Slides were again washed in TBS, and sections were stained with 0.3% Sudan Black B to minimize autofluorescence. After rinses in TBS and then phosphate buffer, coverslips were applied using antifade mounting solution (Dako, Carpinteria, CA). The slides were stored in the dark. Kisspeptin and PR-immunoreactive (ir) cells were identified under fluorescent illumination, with a single observer counting the total number of cells stained. For each ewe, the degree of double labeling was calculated as a percentage of the total number of kisspeptin-ir cells and then averaged across animals to produce a mean (± SEM).

Statistical analysis

All data are expressed as the means (± SEM), and statistical analysis was by one-way ANOVA. Two-way ANOVA was initially employed to determine the treatment effects in the rostral, medial, and caudal divisions of the ARC. The level of $P < 0.05$ was considered significant and the least significant differences test was used as a *post hoc* test.

Results

Distribution of KiSS-1 mRNA in the hypothalamus of the ewe

Cells expressing KiSS-1 mRNA were readily identifiable in the ARC (Fig. 1), and a smaller population was also seen in the POA. No KiSS-1-expressing cells were found in any other neighboring regions of the hypothalamus.

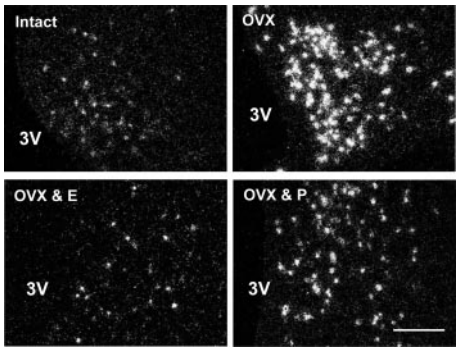

FIG. 1. Dark-field photomicrographs showing representative sections of the middle ARC and KiSS-1 mRNA-expressing cells (as shown by the presence of silver grain clusters) from gonad-intact, OVX, OVX plus E, and OVX plus P ewes. 3V, Third ventricle. *Scale bars*, 200 μm.

Experiment 1: effect of ovariectomy and E or P replacement on KiSS-1 expression

In the ARC, ovariectomy increased the number of cells expressing KiSS-1 mRNA by over 3-fold ($P < 0.001$) and increased the level of expression per cell by 40% ($P < 0.05$) compared with intact (luteal) controls (Figs. 1 and 2). Treatment with E negated the effect of ovariectomy in terms of cell number ($P < 0.001$ compared with OVX) and expression per cell ($P < 0.05$ compared with OVX). Treatment of OVX ewes with P reduced the number of KiSS-1 mRNA-expressing cells by 37% ($P < 0.01$ compared with OVX) but not to the same extent as E treatment. Hence, the number of KiSS-1-expressing cells in the ARC of OVX plus P animals was 2-fold higher than the level in the ARC of luteal-phase animals ($P < 0.05$). P treatment also diminished the effect of ovariectomy on KiSS-1 mRNA per cell ($P < 0.05$ compared with OVX). The change in the level of expression with E or P treatment was uniform across the entire ARC, with no difference between rostral, medial, and caudal divisions. There was no difference between groups in the number of KiSS-1 mRNA-expressing cells or the level of expression of KiSS-1 in the POA (Fig. 2), although the effect of E appeared to reduce the number of cells (but not expression per cell). It should further be noted that the number of cells in the POA of OVX animals was one tenth that of the ARC and the direction of change in the data for the POA was not the same as that seen in the ARC.

Experiment 2: kisspeptin/PR coexpression in the ARC

Cells expressing PR were found throughout the ARC, and the majority of kisspeptin-ir cells colocalized with PR (Fig. 3). Quantitative analysis indicated that 86 ± 3% of all kisspeptin-ir cells also immunostained for PR.

Experiment 3: seasonal expression of KiSS-1 mRNA expression

In the ARC, the number of cells expressing KiSS-1 mRNA was over 2-fold higher in February than in December ($P < 0.001$; Figs. 4 and 5). The number KiSS-1-expressing cells fell between February and May, although this did not reach statistical significance. Expression was 44% lower in October than in February ($P < 0.01$). The level of KiSS-1 expression per cell did not change across the year (Fig. 5). There was no effect of season on KiSS-1 expression in the POA, even though the trend was similar to that seen in the ARC (Fig. 5).

Discussion

We have shown that kisspeptin neurons in the ARC of the ewe brain are regulated by both E and P and are targets for P and that these kisspeptin neurons also display steroid-independent seasonal regulation. These data are consistent with previous reports in the mouse and rat, in which KiSS-1 mRNA in the ARC was found to be regulated by gonadal steroids (9, 17, 26, 27). In the ewe, KiSS-1 expression in the ARC was up-regulated by ovariectomy and corrected with E replacement, but physiological replacement with P only partially countered the effect of ovariectomy. Furthermore, the actions of these sex steroids on KiSS-1 appears to be direct,

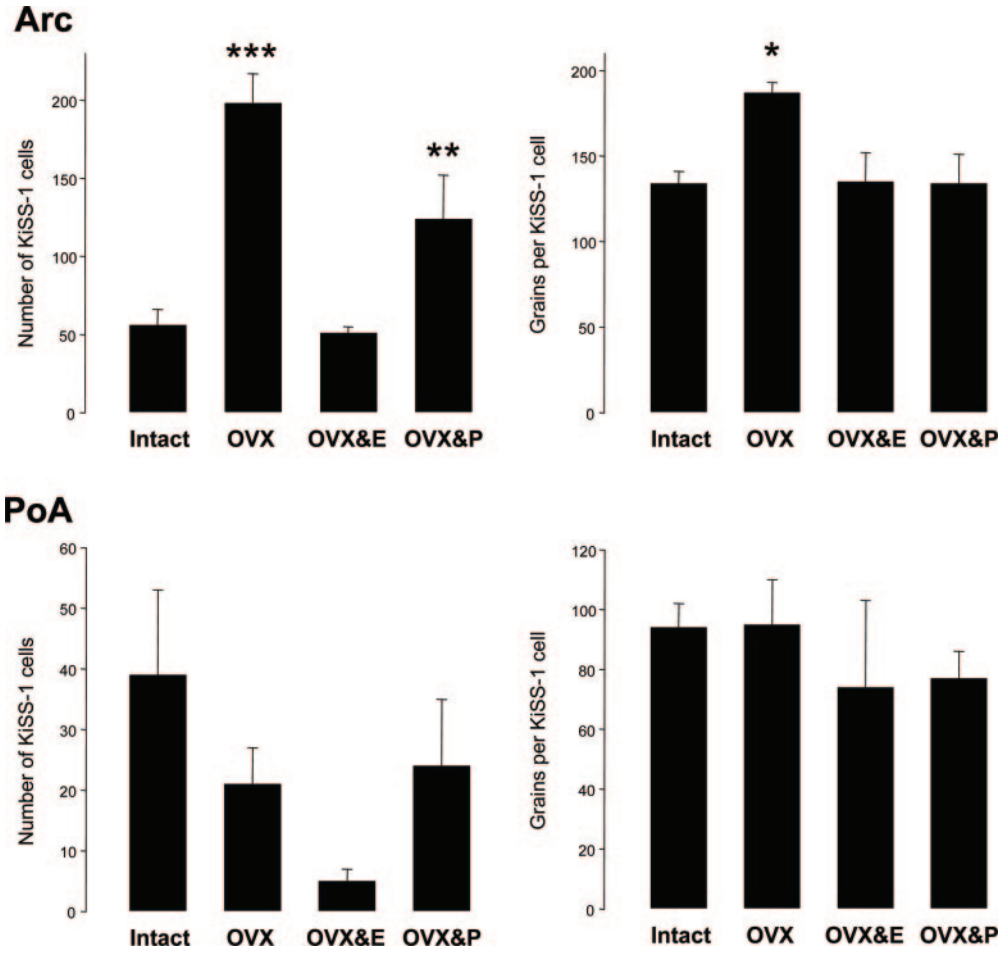

FIG. 2. Effect of ovariectomy and sex-steroid replacement on KiSS-1 expression in the ARC and the POA of the brain of the ewe. The number of KiSS-1 mRNA-expressing cells and the number of silver grains per KiSS-1 cell (reflecting expression per cell) is represented for animals in the luteal phase of the estrous cycle (intact), OVX, and in OVX animals given either E or P replacement. Values are means (± SEM). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared with intact.

because the presence of ERα in kisspeptin-ir neurons of the ARC of the ewe has been established previously (16), and we show that most KiSS-1 neurons express PR. Thus, KiSS-1 neurons in the ARC of the ewe are plausible candidates as mediators of the negative-feedback effects of gonadal steroids on GnRH neurons.

Steroid-responsive cells of the ARC of the ewe brain that project to the medial POA have been implicated in the feedback regulation of GnRH cells (42, 43). The mediobasal hypothalamic region of the ewe brain is recognized as the site at which E acts to evoke a positive-feedback effect on GnRH/LH secretion (30, 44), so the location of KiSS-1-ex-

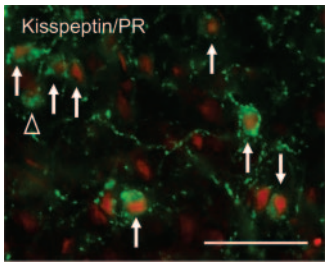

FIG. 3. Representative photomicrograph showing costaining of cells for kisspeptin and PR in the ARC of the ewe brain. Kisspeptin-ir cells are *green* and PR-ir cells are *red*. *Arrows* indicate coexpressing cells. The *open arrowhead* indicates a kisspeptin-positive PR-negative cell. *Scale bar*, 50 μm.

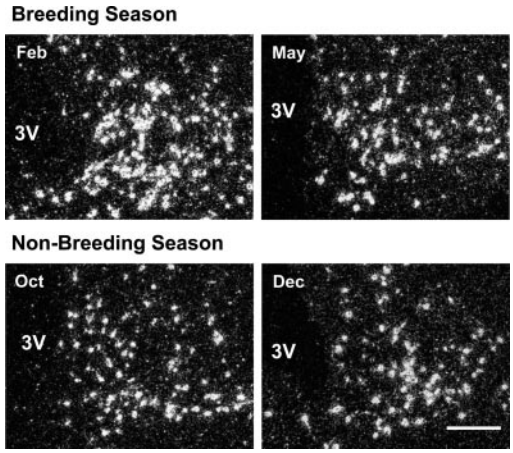

FIG. 4. Dark-field photomicrographs showing KiSS-1 mRNA-expressing cells (as shown by the presence of silver grain clusters) in representative sections of the middle ARC of OVX ewes at four times of the year. 3V, Third ventricle. *Scale bars*, 200 μm.

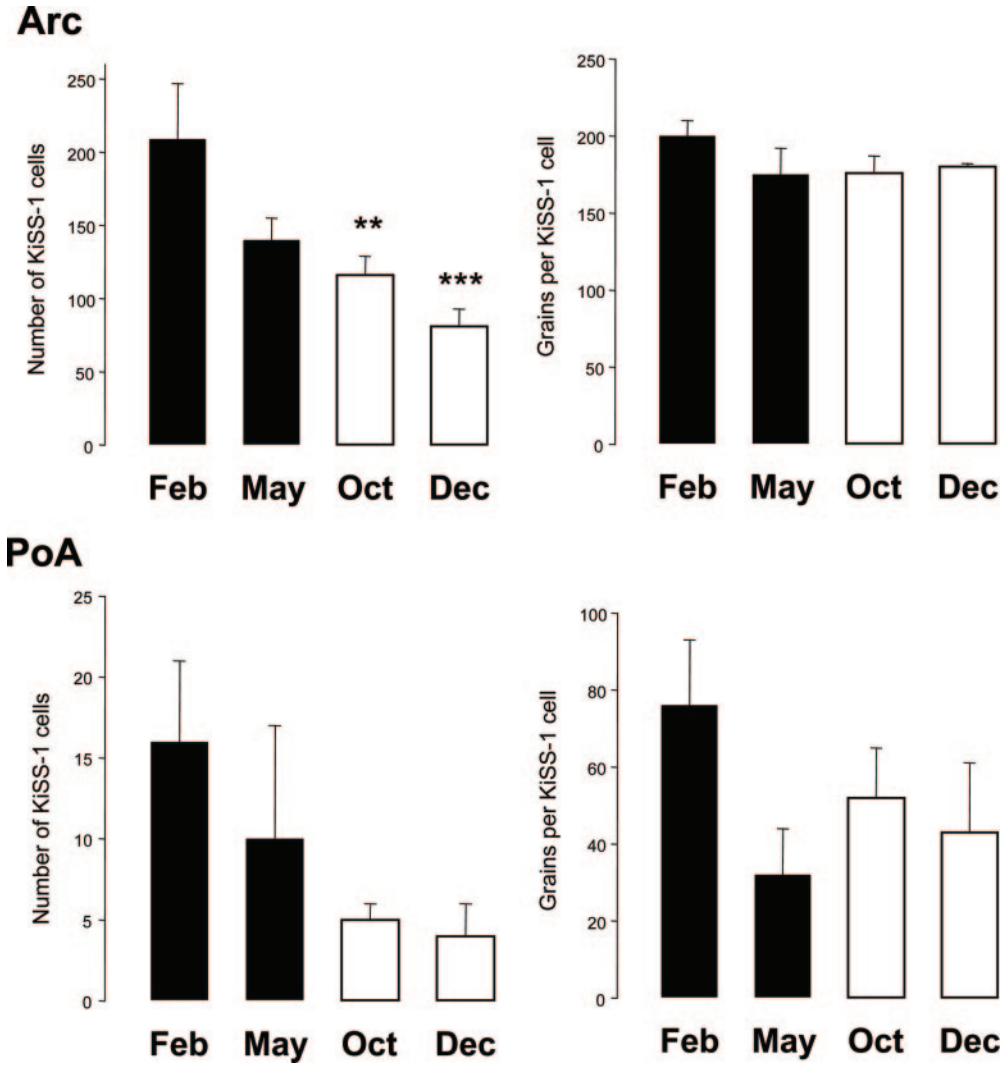

FIG. 5. The number of KiSS-1 mRNA-expressing cells and the number of silver grains per cell (reflecting expression per cell) in the ARC and POA of OVX ewes at four times of the year. *Black bars* represent the breeding season. Values are the means (± SEM). **, $P < 0.01$; ***, $P < 0.001$ compared with February.

pressing cells in the ARC is highly relevant in this regard. Consistent with a major role of kisspeptin in the regulation of the reproductive cycle, we recently reported that KiSS-1 expression in the ARC of the ewe is up-regulated immediately before, and during, the preovulatory GnRH/LH surge, suggesting involvement in the positive-feedback event (15). The present observations suggest that kisspeptin also participates in the relay of negative-feedback regulation of GnRH cells. Interestingly, it appears that only the KiSS-1 neurons located at the caudal portion of the ARC are activated just before the GnRH/LH surge, although a population in the rostral ARC seems to be recruited during the surge (15). The data of the present study indicate that KiSS-1 neurons across the entire ARC are responsive to ovariectomy and steroidal manipulations. This leads us to the enticing proposition that distinct regions of KiSS-1 neurons in the ARC are involved in the negative and positive regulation of GnRH. It remains to be determined whether the kisspeptin-producing cells of the ARC of the ewe brain provide direct input to GnRH cells, which is a focus of attention in our laboratory at present. Such studies may identify the subpopulations that are responsible for the different modes of feedback.

In rodents, KiSS-1 expression in the AVPV appears to be critical in the production of the preovulatory LH surge (27), but this region of forebrain is void of kisspeptin-producing cells in the ewe (15, 16). Nevertheless, KiSS-1 mRNA-expressing and kisspeptin-ir cells are found in the POA of the ewe (15, 16). Using an antibody from Phoenix Pharmaceuticals Inc. (Belmont, CA), a high degree of colocalization of kisspeptin and GnRH was found in the cells of the POA (18), although a different antibody (Caraty no. 566) did not show the same (16). The possibility of a lack of specificity of the Phoenix antibody, at least in our hands, is under active investigation. In the present study, we used the Caraty antibody. The *in situ* hybridization data of the present study complement earlier immunohistochemical data, confirming that kisspeptin is produced in cells found in the POA of the ewe brain. Our data show that KiSS-1 mRNA expression in

the POA is not regulated by either E or P, because differences between groups did not reach statistical significance. Despite this, 50% of kisspeptin-ir cells in the POA express ERα (16).

Many studies have shown that cells within the mediobasal hypothalamus of the ewe are substantially involved in the negative and positive feedback effects of E and P on GnRH secretion (42, 43, 45–47). Although subsets of a wide range of cell types express ER and/or PR, the proportion of these cells that possess steroid receptors is generally low, with the exception of glutamatergic and dynorphin neurons (47, 48). Thus, it is of major significance that virtually all kisspeptin-producing cells in the ARC express ERα, and we have now shown that a very high percentage also express PR. Other studies indicate that there is a high level of coexpression of ERα and PR in the hypothalamus of the ewe (49), so the result is not surprising. It is possible that the glutamate/ERα cells in the ARC of the ewe brain are the same as those that produce kisspeptin. Irrespective of what other transmitters/neuropeptides are found in the kisspeptin-producing neurons, the possession of the relevant steroid receptors and the demonstration of regulation of KiSS-1 expression by E and P suggests that these cells could be integrally involved in feedback regulation of the reproductive system.

In our study, we demonstrate that P treatment was unable to fully suppress KiSS-1 mRNA expression in the ARC of OVX ewes. This result is surprising given that P has been shown to exert a strong negative feedback effect on LH secretion (50, 51) by action at the level of the mediobasal hypothalamus (52). It is possible that the lesser effect of P on KiSS-1 expression in OVX ewes may be due to reduced PR expression in the absence of E (53). In OVX ewes, the suppressive effects of P on LH secretion are eventually lost in the continued absence of E (51). Moreover, E treatment is known to increase the number of PR-expressing neurons in the rat brain (53). The mediobasal hypothalamus is the major site of P negative feedback in the ewe (52), and we have shown that kisspeptin cells located here express a level of PR greater than that seen in cells that produce dynorphin (54), β-endorphin (54), tyrosine hydroxylase (42), and neuropeptide Y (42). Furthermore, these aforementioned cells are known to project from the ARC to GnRH-rich regions of the ovine POA (42, 54). We show that KiSS-1 expression at the ARC is regulated by P, leading us to conclude that kisspeptin cells are most likely involved in the transmission of P feedback to GnRH cells, whether this is through direct or indirect input to the latter. P does not exert such negative feedback when implanted into the POA (52), so we did not examine kisspeptin/PR coexpression at this level. It will be instructive to ascertain whether the kisspeptin cells of the ARC project directly to GnRH cells in the ovine brain, and appropriate neural tracing studies are in progress.

Sheep are seasonally breeding mammals, with an annual cycle of reproductive function that is controlled by photoperiod. This is reflected in alterations in GnRH and LH secretion that are regulated by steroid-dependent and steroid-independent mechanisms (31–35). In the present study, we investigated the steroid-independent mechanism by examining the expression of KiSS-1 in OVX ewes at different times of the year, which represented the breeding and the nonbreeding seasons. We showed a marked elevation of KiSS-1 mRNA in the ARC between the end of the anestrous season (December) and the onset of the breeding season (February). This change in KiSS-1 expression may act as a neuroendocrine switch allowing the onset of the breeding season, in a similar fashion to that recently proposed for the onset of puberty in the mouse (8). Expression of KiSS-1 in cells localized in the ARC of the subhuman primate also increases across puberty onset (14). If, as has been suggested (55, 56), the transition from the anestrous season to the breeding season represents a similar mechanism, this could provide a model relevant to puberty. We do not know whether the seasonal shift in the sensitivity of the reproductive neuroendocrine axis to E negative feedback also involves a further alteration in KiSS-1 expression, but appropriate studies are in progress.

The frequency of pulsatile episodes of LH secretion (reflecting GnRH secretion) is reduced during anestrus in OVX ewes (31–33), and this may be a direct function of reduced kisspeptin action. To test this hypothesis, it will be necessary to measure GnRH/LH secretion in response to kisspeptin infusion at different stages of the circannual breeding cycle. One might hypothesize that there is a seasonal alteration in responsiveness to kisspeptin in terms of GnRH/LH secretion. Because there is no morphological difference in GnRH neurons between the breeding and nonbreeding season (57), it appears that afferents regulating GnRH release would be of critical importance to this phenomenon. Indeed, synaptic inputs on to ovine GnRH cells undergo marked seasonal rearrangements that are independent of gonadal steroid hormones and may reflect the intrinsic seasonality of the brain (58, 59). It is possible that there is some seasonal alteration in the degree to which kisspeptin provides input to GnRH cells.

Similar results to ours have been seen in the hamster (60), which is a long-day breeder. Accordingly, in this species, limited data indicate that KiSS-1 expression is higher under long-day photoperiod than under short days. The question remains as to what the regulatory stimulus might be for the change in KiSS-1 expression. One possibility is that melatonin signaling alters function of the KiSS-1-expressing cells, because melatonin receptors are found in the basal hypothalamus (61) and melatonin acts at this level in the ovine brain (61–63). Alternatively, it may be that the seasonal alteration in KiSS-1 expression is related to a change in nutrition (64). Certainly there is a link between nutrition and kisspeptin, because mice lacking leptin (*ob/ob* mice) have down-regulated KiSS-1 expression (65) and other work in rodent models shows that expression is regulated by nutritional manipulation (66, 67).

In summary, we show that KiSS-1 neurons in the ARC of the brain of the ewe are regulated by chronic E or P treatment, suggesting a that these cells participate in the negative-feedback control of GnRH secretion. This effect is most probably due to direct steroid action on kisspeptin-producing cells, because they possess the relevant receptors. In contrast, the KiSS-1-expressing cells of the POA are not significantly affected by gonadectomy or E or P replacement. We also report that KiSS-1 mRNA expression is up-regulated between the end of the anestrous season and the beginning of the onset of the breeding season, indicating that kisspeptin may be

involved in the activation of central mechanisms controlling seasonal changes in reproductive function.

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