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Colocalization of Kisspeptin and Gonadotropin-Releasing Hormone in the Ovine Brain

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Kisspeptin is a peptide that has been implicated in the regulation of GnRH cells in the brain. Immunohistochemical studies were undertaken to examine the distribution of kisspeptin-immunoreactive (IR) cells in the ovine diencephalon and determine the effect of ovariectomy in the ewe. We report that kisspeptin colocalizes to a high proportion of GnRH-IR cells in the preoptic area, which is a novel finding. A high level of colocalization of kisspeptin and GnRH was also seen in varicose neuronal fibers within the external, neurosecretory zone of the median eminence. Apart from the kisspeptin/GnRH cells, a population of single-labeling kisspeptin-IR cells was also observed in the preoptic area. Within the hypothalamus, kisspeptin-IR cells were found predominantly in the arcuate nucleus, and there was an increase in the number of immunohistochemically identified cell within this nucleus after ovariectomy. Kisspeptin-IR cells were also found in the periventricular nucleus of the hypothalamus, but the number observed was similar in gonad-intact and ovariectomized ewes. The colocalization of GnRH and kisspeptin within cells of the preoptic area and GnRH neurosecretory terminals of the median eminence suggests that the two peptides might be cosecreted into the hypophyseal portal blood to act on the pituitary gland. Effects of ovariectomy on the non-GnRH, Kisspeptin-IR cells of the hypothalamus suggest that kisspeptin production is negatively regulated by ovarian steroids. (*Endocrinology* 147: 804–810, 2006)

PULSATILE SECRETION of GnRH into the hypophyseal portal blood drives the synthesis of the gonadotropins, LH, and FSH in the pituitary gland, and there is tight coupling between the secretory bursts of GnRH and LH (1). GnRH cells do not express estrogen (E) receptor (ER)-α or progesterone receptor but do express ERβ (2–4), and feedback regulation by gonadal steroids is effected via interneuronal circuits within the brain (1). ERβ appears to play only a minor role in the regulation of reproduction (5–7), perhaps via a nongenomic steroid receptor pathway (8, 9). Thus, it is generally accepted that cells in the brain that possess the appropriate steroid receptors mediate the feedback effects of steroid hormones, with other brain systems mediating effects of season, stress, and nutrition on the reproductive axis (10). Kisspeptin is a newly identified peptide that has been found in the brain and appears to play an important role in the regulation of reproductive function. Cells that produce kisspeptin may relay steroid feedback effects and other modulatory influences on GnRH synthesis and secretion.

Kisspeptin-54, -14, -13, and -10 are active forms of the 145-amino acid translation product encoded by the KiSS-1 gene (11–15). Kisspeptins are ligands for the previously described orphan G protein-coupled receptor 54 (GPR54) (11–14). Because a mutation in this receptor causes hypogonadotropic hypogonadism, kisspeptins are thought to play a role in the pubertal activation and GnRH neuronal activity in humans, primates, and mice (16–19). Expression of the KiSS-1 gene and its proteins has been demonstrated in the brains of rats, mice, monkeys, and humans, especially in the hypothalamus and medulla oblongata (11, 20–23). The GPR54 receptor is also found in the hypothalamus, cerebellum, pituitary, and spinal cord (11, 19). Intracerebroventricular (icv) infusion or iv injection of kisspeptin-10 or -54 increases LH and FSH secretion in prepubertal and adult male and female rats (24–27), adult male mice (20), and sheep (28). Furthermore, treatment of male mice (20), rats (27), and agonadal juvenile monkeys (23) with a GnRH antagonist prevented the effect of kisspeptin-54 on LH and FSH secretion, which was taken to mean that the effect of kisspeptin is via the release of GnRH. Further evidence in support of a stimulatory action GnRH secretion was provided by studies showing that icv-administered kisspeptin increased GnRH levels in the cerebrospinal fluid within the third cerebral ventricle (3V) of the sheep brain (28).

Other recent studies suggest that kisspeptin function is controlled by steroid hormones because the kisspeptin-producing cells of the medial basal hypothalamus of rats and mice express either ERα and ERβ, androgen receptor, or both (29–31), and castration leads to increased levels of KiSS-1 mRNA expression in hypothalamus of several species including rats (25, 32), mice (29, 30), and monkeys (23). Furthermore, steroid hormone replacement reduced the level of KiSS-1 mRNA expression in the arcuate nucleus (ARC) of ovariectomized (OVX) rats and increased expression in the ventromedial nucleus (VMH) (33). Similar results were obtained in castrated male mice (30) and OVX female mice (29) after E replacement. In contrast, a KiSS-1 mRNA expression in anteroventral periventricular region of the brain is re-

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Abbreviations: ARC, Arcuate nucleus; dbB, diagonal band of Broca; E, estrogen; ER, E receptor; GPR54, G protein-coupled receptor 54; icv, intracerebroventricular; IR, immunoreactive; OVX, ovariectomized; PB, sodium phosphate buffer; PeriVH, periventricular nucleus of the hypothalamus; POA, preoptic area; TBS, Tris-buffered saline; 3V, third cerebral ventricle; VMH, ventromedial nucleus.

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duced in mice of both sexes after castration, and this effect is reversed by E replacement (29, 30). Collectively, all of the aforementioned studies suggest that gonadal steroids regulate kisspeptin production and further suggests that kisspeptin regulates the reproductive axis via GnRH cells.

Because the sheep is an important species for neuroendocrine research and there is evidence of evoked GnRH release by kisspeptin in this species, further investigation of this species was appropriate. We examined the distribution of kisspeptin in the diencephalon of the ovine brain and found that it colocalizes with GnRH cells in the diagonal band of Broca (dbB) and preoptic area (POA). Second, in view of the evidence that the kisspeptin system is regulated by gonadal steroids in other species, we investigated whether the number of kisspeptin-immunoreactive (IR) was affected by ovariectomy.

Materials and Methods

Ethics

The experimental procedures were in accordance with the requirements established by the Australian Prevention of Cruelty to Animals Act 1986, and prior approval for experimental procedures was granted by the ethics committees of Monash Medical Centre and Victorian Institute of Animal Science. In addition, all efforts were made to minimize the number of animals used and their suffering.

Animals

Adult Corriedale ewes of similar age (5–6 yr) and weight were used. Brains of ovary-intact animals were collected during the luteal phase of the estrous cycle and those of OVX ewes were collected 4 wk after ovariectomy. The estrous cycles of ovary-intact animals were synchronized by im injection of 125 μg of the synthetic luteolysin, Cloprostenol (Estrumate, Pitman-Moore, Sydney, New South Wales, Australia) (34, 35), and brains were collected on d 10 of the ensuing estrous cycle (luteal phase). Animals were injected (iv) with 25,000 IU of heparin and killed 5 min later by iv injection of 20 ml of Lethobarb (Virbac, Peakhurst, Victoria, Australia). The heads of the sheep were perfused with 2 liters of heparinized saline followed by 1 liter of Zamboni's fixative, containing 4% paraformaldehyde plus 15% picric acid in 0.1 M sodium phosphate buffer (PB) (pH 7.3) and 0.5 liter of the same fixative solution containing 20% sucrose. In addition, for comparison, adult male rats, Sprague Dawley (n = 2), were anesthetized by ip injection of 40 mg/kg of sodium pentobarbital (Rhone Merieux, Brisbane, Queensland, Australia) and then perfused via the left ventricle of the heart. The perfusion consisted initially of 40 ml 0.9% saline followed by 40 ml of 4% paraformaldehyde in PB. The brains were removed and placed in a solution of 0.1 M PB containing 30% of sucrose until they sank and were then frozen in powered dry ice. Coronal sections (40 and 30 μm, respectively, for sheep and rat brains) were cut from blocks of the POA and hypothalamus using a cryostat, and the sections were collected into tissue culture wells containing cryoprotectant (36) and stored at −20 C until used.

Colocalization of GnRH and kisspeptin in sheep and rat dbB/POA

Immunohistochemistry was performed on free-floating sections of dbB/POA of intact and OVX ewes as described previously (37). Sets of 12 sections were taken at intervals of 240 μm extending from the rostral dbB to the caudal POA, as represented in Fig. 1, A–D, of Lehman *et al.* (38). The sections were initially rinsed in 0.1 M Tris-buffered saline (TBS; pH 7.4) and incubated for 20 min with 1% sodium borohydride (Sigma-Aldrich Corp., Melbourne, Victoria, Australia) in 0.1 M TBS (pH 7.4), rinsed, and incubated overnight at 4 C with blocking buffer containing 10% normal goat serum plus 0.3% Triton X-100 (Sigma-Aldrich) in TBS. The sections were then incubated for 72 h at 4 C with a cocktail containing a rabbit polyclonal antibody against kisspeptin-10 (Metastin 45–54-amide of the human sequence) (Phoenix Pharmaceutical Inc., Belmont, CA) at a dilution of 1:2000 and a monoclonal antibody against GnRH (HU11B courtesy of Prof. Urbanski, Oregon Regional Primate Research, Beaverton, OR), at a dilution of 1:2000. After washing in TBS, they were incubated with a mixture of goat antirabbit Alexa 488 and goat antimouse Alexa 546 (Molecular Probes Inc., Eugene, OR) (dilution 1:500) for 1 h. Finally, the sections were washed in 0.1 M PB and mounted on gelatinized slides and dried for 2 h at room temperature. To minimize autofluorescence, the sections were stained with 0.3% Sudan Black B (39) and coverslips were applied using an antifade mountant (Dako, Sydney, New South Wales, Australia). The slides were stored in the dark at 4 C until analysis. A single observer, blind to the treatment group, counted the total number of kisspeptin-IR, GnRH-IR, and double-labeled cells in each section.

Sections of rat brain representing the POA were mounted on SuperFrost slides and left overnight to dry. Antigen retrieval was performed using 0.01 M TBS (pH 9.0) in a microwave oven at 1000 W (2 × 5 min). The slides were then left to cool and rinsed in TBS and then incubated with a mixture of kisspeptin and GnRH antibodies as described above for ewe brain sections.

Kisspeptin-IR cells and varicose nerve fibers in the ovine brain and the effect of ovariectomy on kisspeptin cells

Sections throughout the hypothalamus (seven per animal) were mounted on SuperFrost slides, subjected to antigen retrieval as for rat brain (see above), and processed for kisspeptin immunohistochemistry. The sections were washed in TBS and incubated overnight with blocking solution containing 0.3% Triton X-100 (Sigma-Aldrich, New South Wales, Australia) in TBS and then incubated with kisspeptin antibody at dilution of 1:2000 for 72 h at 4 C. Antibody labeling was revealed by incubating with goat antirabbit Alexa 488 (Molecular Probes; dilution 1:500) for 1 h.

Initial tests showed that kisspeptin-IR cells in the hypothalamus were variably immunostained for kisspeptin, perhaps due to variable levels of peptide within the cells of these regions. This was not the case in the POA, in which kisspeptin immunostaining was generally stronger, with little evidence of weakly staining cells. Accordingly, we used antigen retrieval for immunohistochemistry in the hypothalamic nuclei, but when we examined the dbB/POA, this method was not used. In the dbB/POA, antigen retrieval did not alter the number of kisspeptin-IR cells observed (data not shown). A single observer counted the total number of kisspeptin-IR cells in each section.

Antibody adsorption tests

The use of the kisspeptin antibody to immunostain cells has been reported previously (22), so extensive specificity tests were not undertaken. To determine whether the kisspeptin antibody cross-reacted with GnRH, the antiserum (1:2000) was incubated overnight at 4 C with either 0.1 or 1 mg/ml GnRH (Auspep, Parkville, Melbourne, Australia) and was then applied to sections of hypothalamus and POA from intact animals for 72 h at 4 C. To further determine specificity, the kisspeptin antibody was incubated with 10 μg/ml of the kisspeptin (molecular weight 1302.44, Phoenix Pharmaceuticals) before application to tissue sections. To determine possible cross-reactivity of the GnRH antibody with kisspeptin, the antiserum (1:2000 dilution) was preabsorbed with 10 μg/ml kisspeptin and then applied to hypothalamic sections. In another series of experiments, the immunohistochemical procedure was carried out without application of primary antibody. All three sets of slides were processed for fluorescence immunohistochemistry.

Photography

To examine single and dual labeling, we used a BMX 50 microscope (Olympus, Tokyo, Japan) equipped with mercury light and appropriate filter systems. Excitation wavelengths were 546 nm for Alexa 546 (Texas Red) and 488 nm for Alexa 488 (FITC); emission wavelengths were 615 nm for Texas Red and 520 nm for FITC. GnRH-IR and kisspeptin-IR cells in the dbB/POA and kisspeptin-IR cells in the medial basal hypothalamus were mapped using a digitizing X-Y plotting system (MD4 plot, AccuStage, Shoreview, MN).

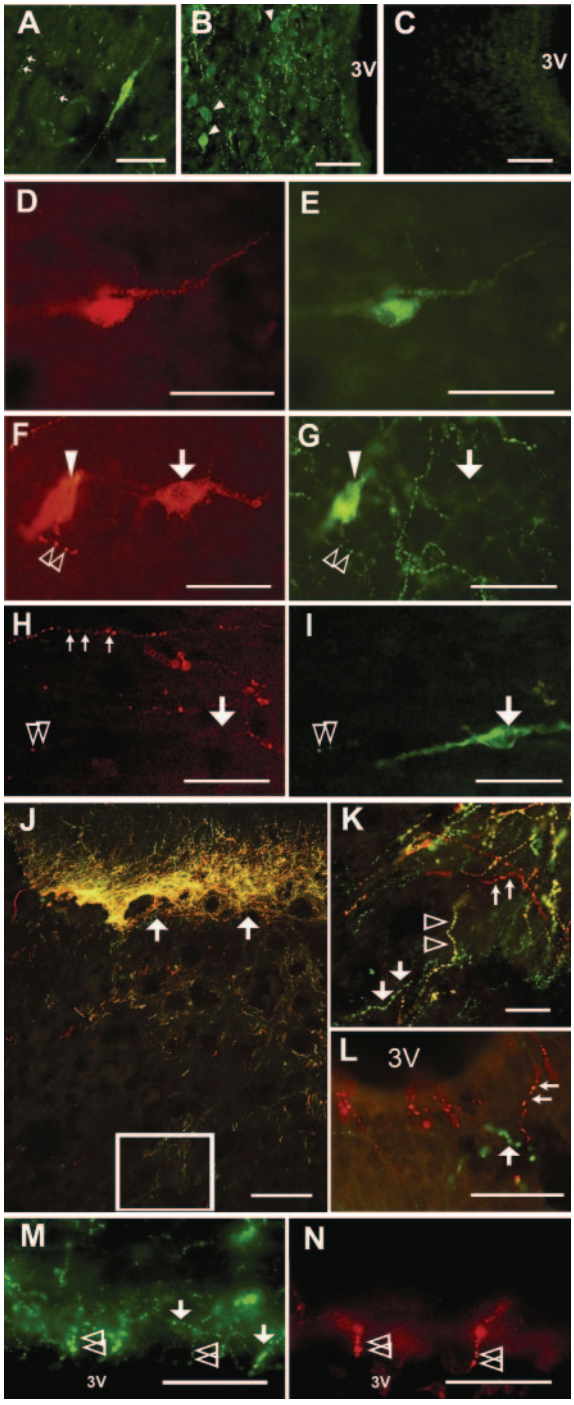

FIG. 1. Photomicrographs of kisspeptin and GnRH immunostaining in the ovine brain and the location of kisspeptin-IR cells in the hypothalamus. In all cases GnRH immunostaining is *red* and kisspeptin immunostaining is *green*. Colocalization of the two peptides is seen in *yellow*. A, A kisspeptin-IR cell in the POA of an ovary-intact ewe after preadsorption of the antiserum with 100 μg/ml of GnRH (*scale bar*, 50 μm). B (*scale bar*, 50 μm), Kisspeptin immunostaining in cells and varicose fibers of the ARC. C (*scale bar*, 50 μm), Lack of immunostaining in an adjacent section incubated with antiserum that was preadsorbed with kisspeptin (10 μg/ml). D (*scale bar*, 50 μm), Immunostaining of a cell in the POA of a ewe with GnRH antiserum. E (*scale bar*, 50 μm), Immunostaining of the same cell with kisspeptin antiserum. F (GnRH immunostaining) and G (kisspeptin immunostaining) (*scale bar*, 50 μm), GnRH cells in a single section that either colocalize (*arrowhead*) or do not colocalize (*arrow*) kisspeptin. *Open arrowheads* indicate a varicose nerve fiber, which was immunoreactive for GnRH (F) and kisspeptin (G). H (GnRH immunostaining) and I (kisspeptin immunostaining) (*scale bar*, 50 μm), Images of the same section showing a varicose GnRH-IR fiber that is not immunoreactive for kisspeptin (*small arrows*) and a GnRH-IR fiber that is immunoreactive for kisspeptin (*arrowheads*). The *arrow* (H and I) indicates a cell that is kisspeptin-IR but not GnRH-IR. J (*scale bar*, 50 μm), Colocalization of GnRH-IR and kisspeptin-IR in the median eminence, and the *arrows* (J) indicate a dense accumulation of neurosecretory terminals in the external zone of the median eminence, in which intense double labeling was seen. K (*scale bar*, 10 μm), Enlargement of the box indicated in J, showing double-labeled fibers (*open arrowheads*), but single-stained GnRH-IR (*small arrows*) and single-stained kisspeptin-IR (*large arrows*) varicose nerve fibers were also present. L (*scale bar*, 20 μm), Image of the POA of a male rat showing GnRH-IR varicose nerve fibers (*small arrows*) and kisspeptin-IR varicose fibers (*large arrows*). M and N (*scale bar*, 50 μm), Images of the ependymal layer of the3V in which kisspeptin-IR (M) varicose fibers also immunostained for GnRH (N; *double arrowheads*); *arrows* indicate kisspeptin immunostaining that is not colocalized with GnRH.

Statistics

Data for the total number of kisspeptin-IR cells per animal in the dbB/POA, ARC, and periventricular nucleus of the hypothalamus (PeriVH) were analyzed using the repeated measures method (version 13, SPSS, Chicago, IL). Estimates of percentages of kisspeptin-IR/GnRH-IR cells in the dbB/POA were subjected to arcsin transformation, as recommended for such data (40) and then analyzed by single-factor ANOVA.

Results

Kisspeptin and GnRH immunolabeling in dbB/POA and median eminence

The ability of the kisspeptin antiserum to immunohistochemically identify cells in the ovine brain was not diminished by preadsorption of the antiserum with 0.1–1 mg/ml GnRH (Fig. 1A). Kisspeptin immunostaining in the ARC (Fig. 1B) was blocked by preadsorption of the antibody with 10 μg/ml kisspeptin (Fig. 1C). Preadsorption of the GnRH antibody with 10 μg/ml kisspeptin did not block immunostaining (data not shown).

Kisspeptin-IR cells and immunoreactive varicose nerve fibers were present in sections of the dbB and POA. A high percentage of GnRH cells colocalized kisspeptin immunoreactivity (Fig. 1, D–G). The mean (±SEM) percentage of GnRH-IR cells that were also immunoreactive for kisspeptin was similar in intact (90 ± 2.3%) and OVX ewes (89 ± 1.8%). A small subpopulation of GnRH-IR cells in the dbB/POA of ovary-intact and OVX ewes (10 ± 2.3 and 11 ± 1.8%, respectively) were not immunoreactive for kisspeptin (Fig. 1, F and G). Figure 2 shows the distribution of kisspeptin-IR, GnRH-IR, and double-labeled cells in the dbB/POA. Cells labeled only for kisspeptin were interspersed among double-labeled cells and GnRH-IR cells with no specific distribution throughout the dbB/POA. Of the total population of kisspeptin-IR cells in the dbB/POA, one fifth were not immunoreactive for GnRH (Fig. 1, H and I) and ovariectomy did not affect this proportion (19.8 ± 2.9% in intact and 19.0 ± 6.8% in OVX).

A dense plexus of kisspeptin-IR varicose nerve fibers was observed in the median eminence, and the majority of immunostaining was colocalized with GnRH (Fig. 1, J and K).

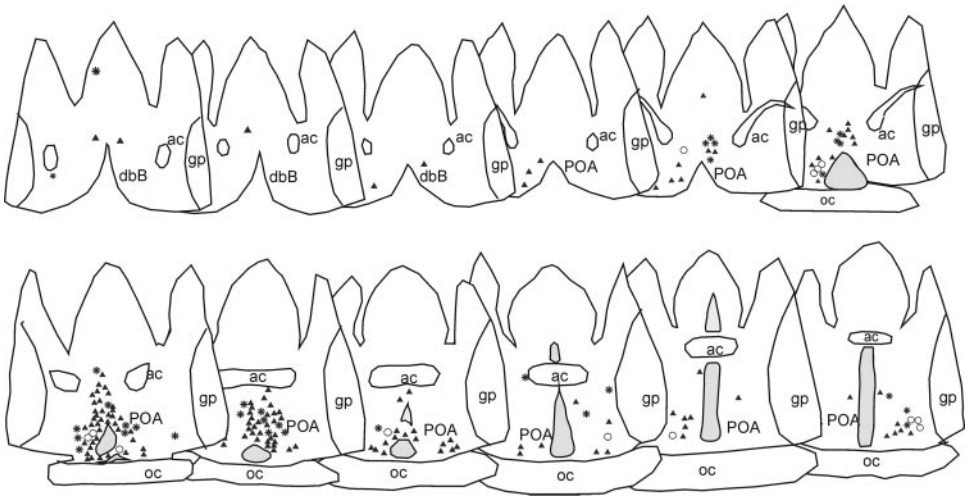

FIG. 2. Diagrammatic representation of the distribution of GnRH and kisspeptin cells in the POA of the ewe brain. *Triangles* indicate cells colocalizing kisspeptin and GnRH, the *circles* indicate cells staining for GnRH alone, and *asterisks* indicate cells staining for kisspeptin alone. ac, Anterior commissure; gp, globus pallidus, oc, optical chiasm. The *shaded area* shows the 3V.

Varicose kisspeptin-IR nerve fibers were also seen between the cells of the ependymal layers of the lateral ventricle and 3V (Fig. 1M), and some of these displayed GnRH immunoreactivity (Fig. 1N). Kisspeptin-IR varicose nerve fibers richly innervated the bed nucleus of the stria terminalis and fibers were seen in the septal area, with occasional kisspeptin-IR cells being found in these areas.

Kisspeptin and GnRH were not colocalized in either cell bodies or varicose nerve fibers of the male rat brain (Fig. 1L).

Kisspeptin-IR cells in the hypothalamus

In the hypothalamus, kisspeptin-IR cells were located predominantly in the ARC, mostly in the region of this nucleus that is lateral to the 3V. Occasional kisspeptin-IR cells were seen in the sub-ventricular region of the ARC (Fig. 3, A and C). Kisspeptin-IR cells were also localized to the PeriVH, adjacent to the 3V and caudal to the paraventricular nucleus (Fig. 3, D and E). A few cells were seen in the dorsomedial nucleus and VMH and the caudal region of the paraventricular nucleus. Kisspeptin-IR varicose nerve fibers richly innervated the hypothalamus including both the ventral and lateral regions of the ARC, PeriVH (Fig. 3A), VMH, and dorsomedial hypothalamus (data not shown). A few immunoreactive fibers were observed in the lateral hypothalamic area.

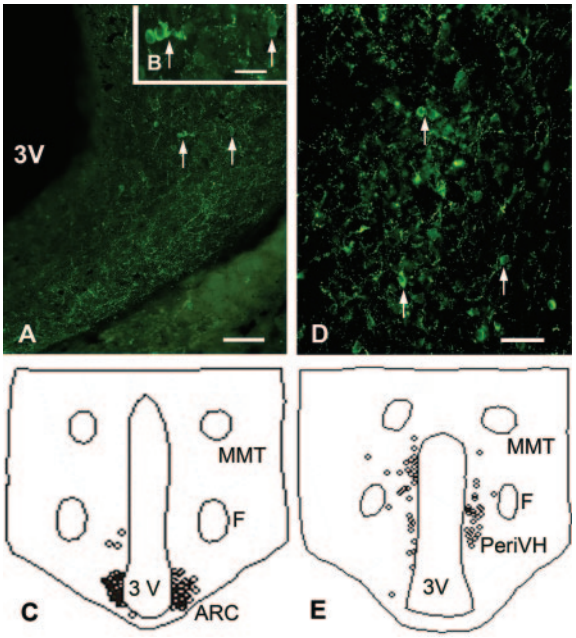

FIG. 3. Kisspeptin distribution in the hypothalamus of the ewe brain. A (*scale bar*, 50 μm), Low-power image of the ARC showing kisspeptin-IR cells. B, Higher-power representation of the cells indicated by *arrows* indicated in A (*scale bar*, 25 μm). C, Diagrammatic representation of kisspeptin-IR cells in the ARC. D, Kisspeptin-IR cells in the PeriVH (*scale bar*, 50 μM). E, Diagrammatic representation the cells in this area. *Arrows* indicate kisspeptin-IR cells. MMT, Mammillothalamic tract; F, fornix.

Significantly more ($P < 0.05$) kisspeptin-IR cells were observed in the ARC of OVX ewes, compared with the number counted in luteal-phase, gonad-intact ewes (Table 1). There was no effect of gonadal status on the number of kisspeptin-IR/non-GnRH-IR cells in the dbB/POA or the number of kisspeptin-IR cells in the PeriVH (Table 1).

Discussion

We report that the majority of GnRH neurons located in the dbB/POA of ovine brain are immunoreactive for kisspeptin, whereas no such colocalization is observed in the adult male rat brain. Colocalization of kisspeptin and GnRH was also observed in varicose nerve fibers within the ovine brain or in the ependyma, organum vasculosum of the lamina terminalis, and neurosecretory zone externa in the median eminence. This novel finding for the ovine brain raises the possibility that kisspeptin might regulate GnRH cells in an autocrine manner and/or may be cosecreted with GnRH into the hypophyseal portal blood to act on the pituitary gland. We further report, for the first time in any species, that kisspeptin peptide levels in the ARC are increased after ovariectomy.

Colocalization of kisspeptin and GnRH was not apparent in the brains of male rats, and this has not been reported in previous studies of species other than the sheep, even though dual immunostaining procedures for both peptides show that kisspeptin-IR varicosities are in close contact with GnRH-IR cells in the rat brain (31). Thus, it appears that the colocalization that we observed may occur in only some species. GPR54 is expressed in the pituitary gland as indi-

TABLE 1. Mean ± SEM total number of kisspeptin-IR in the ARC and the PeriVH of the hypothalamus of gonad-intact ewes and OVX ewes

ARC		PeriVH		POA (non-GnRH)	
Gonad intact	OVX	Gonad intact	OVX	Gonad Intact	OVX
120 ± 22.7	243.5 ± 53.3[a]	254.0 ± 36.6	218.7 ± 42.6	38.8 ± 8.1	33.7 ± 1.1

Only the non-GnRH/kisspeptin-IR cells of the POA are included in this analysis.
[a] $P < 0.05$ compared with gonad-intact, luteal-phase ewes.

cated by PCR studies (11, 12),and the possibility exists that kisspeptin is released into hypophyseal portal blood to act on pituitary cells. Alternatively, although not exclusive of the above, kisspeptin in GnRH cells could have an autocrine action to stimulate GnRH secretion because central administration of kisspeptin to sheep increased GnRH levels in the cerebrospinal fluid of the 3V (28). Various studies have shown that icv infusion or iv injection of kisspeptin-10 or kisspeptin-54 increases the secretion of LH in prepubertal and adult male and female rats and adult male mice (24, 25), an effect that is thought to be effected by stimulation of GnRH release (20, 23–28). Further evidence that kisspeptin acts centrally is seen in studies in which iv (41) or sc (27) administration induced Fos protein in GnRH neurons and Fos protein expression is seen in kisspeptin-IR cells of the hypothalamus during the proestrus in rats (31).

Consistent with an action of kisspeptin to stimulate GnRH secretion, GPR54 is expressed by GnRH neurons of cichild fish (42), rat (41), monkey (18), and mouse (28). Whether the same is true for sheep remains to be determined. In rodent species at least, kisspeptin cells appear to provide input to GnRH cells (31), and the general notion is that kisspeptin regulates the GnRH cells by synaptic input. Our data suggest, however, that this is not a general rule and that kisspeptin regulation of GnRH cells may also be affected by an autocrine mechanism. GnRH neurons are interconnected (43), and this is thought to facilitate coordinate firing of the cells for generation of pulsatile discharge of the peptide from terminals in the median eminence. Kisspeptin could participate in such coordinate activity. Further indication that the means by which kisspeptin stimulates reproductive function is via release of GnRH are the observations that pretreatment with GnRH antagonist reduces the gonadotropic response to kisspeptin (20, 27). This, however, does not rule out the possibility that kisspeptin acts at the level of the gonadotrope because antagonist treatment blocks action of GnRH and the pulsatile secretion of LH is entirely dependent on the secretagogue. Thus, application of a test substance (kisspeptin) while blocking of action of the primary secretagogue (GnRH) does not necessarily exclude the possibility that the former acts at the level of the pituitary gonadotrope. Kisspeptin could act in concert with GnRH to regulate secretion of gonadotropins from the pituitary gonadotropes.

Studies of the mouse (29, 30) and rat (31) brain indicate that kisspeptin-producing cells express ERα, supporting the notion that these cells convey E feedback signals to GnRH cells. Participation of these cells in the feedback effects of gonadal hormones is also indicated by the study of KiSS-1 mRNA expression after gonadectomy and steroid hormone replacement in both sexes (23, 25, 29, 30, 32, 33, 41) (see introductory text). In the present study, we used immunohistochemistry to examine the effect of ovariectomy on levels of kisspeptin peptide in the ewe. The effect of ovariectomy was confined to the ARC in which an increase the number of kisspeptin-IR cells was observed. A similar effect was not seen in other populations of kisspeptin-IR cells in dbB/POA and PeriVH. These data corroborate the results obtained for gene expression in rodent species, so there is strong evidence that kisspeptin production in the ovine brain is negatively regulated by gonadal factors, probably steroids.

Because the secretion of GnRH and gonadotropins is increased after ovariectomy (44) and kisspeptin stimulates GnRH secretion into third ventricular cerebrospinal fluid in the sheep (28) and because E acts in the region of the ARC/VMH to cause the preovulatory surge in GnRH/LH secretion (45, 46), it is possible that kisspeptin cells found in the ARC of the ewe brain participate in the positive feedback phenomenon. E does not act within the POA to activate the positive feedback effect in this species (45, 46), so the fact that gonadectomy did not affect the number of immunostaining cells in this region suggests that the steroid feedback via kisspeptin cells is confined to the ARC. The increased Fos expression in kisspeptin cells in the rat brain during proestrus is consistent with the notion that this peptide mediates the positive feedback effect of E (31). Cells of the ARC are rapidly activated by E in the ewe, as indicated by Fos appearance within 1 h (47). Furthermore, E-responsive cells of the ARC and VMH project to the bed nucleus of the stria terminalis and POA (48, 49), so there is a possibility that this pathway involves kisspeptin cells. Apart from the involvement of kisspeptin in steroid feedback to GnRH cells within the brain, the fact that the peptide is produced in GnRH cells and is found in the terminal fields in the median eminence suggests a separate mechanism by which reproductive function may be regulated. The sheep is an ideal model to investigate whether there is cosecretion of GnRH and kisspeptin because hypophyseal portal samples can be obtained with ease (50), and direct pituitary effects on gonadotropes may be investigated *in vivo* (51). Furthermore, the colocalization of the two peptides that we have observed in the sheep brain may also occur in other nonrodent species, and investigation of this is warranted.

The genes for KiSS1 and GPR54 have been cloned for the human, nonhuman, primate, rat, and mouse (11, 12, 14, 18, 52, 53). The ovine homologues for these genes have been reported recently (GenBank accession no. DQ059506) and have relatively low homology with the other species. Because these genes have become available only very recently, we have not yet expanded our studies to the genomic level, but such work is in progress. It will be of interest to ascertain whether the KiSS-1 and GPR54 genes are regulated by steroids as in other species (25, 29, 30, 41).

In conclusion, we report that kisspeptin and GnRH colocalize in cells and neurosecretory terminals in the ovine brain. We also report that ovariectomy increases the number of kisspeptin-IR cells in the ARC of the ewe brain, suggesting that the production of this peptide is regulated by ovarian hormones, probably steroids. Further investigation of the role of kisspeptin in this species and other nonrodent species will elucidate the function of this peptide in the control of reproduction.

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The authors have nothing to declare.

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