Kisspeptin Cells in the Ewe Brain Respond to Leptin and Communicate with Neuropeptide Y and Proopiomelanocortin Cells

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Kisspeptin stimulates reproduction, and kisspeptin cells in the arcuate nucleus (ARC) express Ob-Rb in the mouse. Herein we report studies in ewes to determine whether kisspeptin cells express Ob-Rb and respond to leptin and whether reciprocal connections exist between kisspeptin cells and proopiomelanocortin (POMC) or neuropeptide Y (NPY) cells to modulate reproduction and metabolic function. *Kiss1* mRNA was measured by *in situ* hybridization in ovariectomized ewes that were normal body weight, lean, or lean with leptin treatment by intracerebroventricular (icv) infusion (4 μg/h, 3 d). *Kiss1* expression in the ARC and the preoptic area was lower in hypogonadotropic lean animals than animals of normal weight, and icv infusion of leptin partially restored *Kiss1* expression in lean animals. Single-cell laser capture microdissection coupled with real-time PCR showed that *Kiss1* cells in the preoptic area and ARC express Ob-Rb. Double-label fluorescent immunohistochemistry showed that reciprocal connections exist between kisspeptin cells and NPY and POMC cells. Accordingly, we treated ovariectomized ewes with kisspeptin (5 μg/h, icv) or vehicle for 20 h and examined *POMC* and *NPY* gene expression by *in situ* hybridization. Kisspeptin treatment reduced *POMC* and increased *NPY* gene expression. Thus, kisspeptin neurons respond to leptin and expression of *Kiss1* mRNA is affected by leptin status. Kisspeptin cells communicate with NPY and POMC cells, altering expression of the relevant genes in the target cells; reciprocal connections also exist. This network between the three cell types could coordinate brain control of reproduction and metabolic homeostatic systems. **(*Endocrinology* 151: 2233–2243, 2010)**

There is now unequivocal evidence that the kisspeptin system stimulates the reproductive axis in rodent species (1) as well as sheep (2), monkeys (3), and humans (4). It is also clear that the kisspeptin cells of the brain respond to metabolic signals and may relay relevant information to GnRH cells because *Kiss1* mRNA is reduced in both prepubertal (4) and adult (5, 6) male and female rats deprived of food. In the mouse kisspeptin cells in the arcuate nucleus (ARC) express the signaling form of the leptin receptor (Ob-Rb) (7), and leptin treatment increases *Kiss1* mRNA when administered to the N6 murine hypothalamic mouse cell line (5). Furthermore, repeated administration of kisspeptin to uncontrolled diabetic rats restores the hypogonadotropic axis (8). Reproductive function can also be restored in hypogonadal lean rats (9) and *ob/ob* mice of low fertility (10, 11) by central and ip administration of kisspeptin. *Kiss1* mRNA is decreased in leptin-deficient *ob/ob* mice and leptin treatment stimulates *Kiss1* mRNA expression (7). In lean hypogonadotropic ovariectomized (OVX) ewes, leptin restores pulsatile LH secretion (12), but the effect of lean condition and leptin treatment on *Kiss1* mRNA has not been studied in this animal model.

The regulatory role of leptin on the kisspeptin system in rodent species suggests a means by which the reproductive axis can be informed of metabolic status, but the central circuits that are involved are not elucidated. Many pep-

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Abbreviations: aCSF, Artificial cerebrospinal fluid; ARC, arcuate nucleus; ER, estrogen receptor; GPR, gonadotropin-inhibiting hormone receptor; IIIV, third ventricular; NPY, neuropeptide Y; Ob-Rb, leptin receptor; OVX, ovariectomized; POA, preoptic area; POMC, proopiomelanocortin.

tides within the brain that are involved in energy balance also act to regulate GnRH secretion (13), such as neuropeptide Y (NPY) and melanocortins derived from the *POMC* gene (13–17). NPY stimulates food intake and inhibits reproduction in the sheep (15), whereas the melanocortins reduce food intake (18) and stimulate reproduction in this species (19). Leptin acts to down-regulate the *NPY* gene expression in animals of normal body weight (20–22) but does not do so in lean animals (23), perhaps because of their need to maintain appetite drive. Altering body weight has been observed to have variable effects on *POMC* gene expression (24, 25). In recent studies, however, we found that *POMC* gene expression was reduced in lean, hypogonadotropic OVX ewes but could be restored to normal with leptin treatment (23). Similarly, leptin positively regulates *POMC* mRNA expression in rodent species (20, 26). On this basis, we hypothesized that melanocortins positively regulate kisspeptin cells. Regarding NPY, a reduction in *Kiss1* gene expression was seen in NPY knockout mice, and *Kiss1* gene expression was stimulated by NPY in hypothalamic N6 cell lines (5), suggesting that NPY may positively stimulate kisspeptin cells. There are no reports implicating the *Kiss1* system in the regulation of POMC and/or NPY expression, nor do data exist regarding the link between metabolic regulators and the kisspeptin system in the sheep.

The aim of the present study was to further interrogate the role of the kisspeptin system in relaying metabolic information to the reproductive axis in the ewe. We tested the hypothesis that the kisspeptin system responds to leptin status. We measured Ob-Rb expression in kisspeptin cells and quantified *Kiss1* mRNA in normal and lean hypogonadotropic animals with and without leptin treatment. We also examined communication of the kisspeptin cells with other cellular systems involved in both metabolic regulation and the reproductive axis, *viz.* the NPY and proopiomelanocortin (POMC) systems of the ARC. Our results show that POMC and NPY cells receive neuronal input from kisspeptin-immunoreactive cells of the ARC and that *POMC* gene expression is reduced and *NPY* gene expression is increased after kisspeptin treatment of OVX ewes. We conclude that the kisspeptin cells of the ovine ARC and preoptic area (POA) express Ob-Rb and respond to leptin. Thus, kisspeptin cells may have dual function to regulate both reproduction and metabolic homeostasis.

Materials and Methods

Animals

Adult Corriedale ewes were maintained on pasture or in feed lots. For experimentation, the animals were housed in individual pens with natural lighting and had access to water *ad libitum*. The animals were conditioned to pen housing and handling for 1 wk before experimentation, and third ventricular (IIIV) cannulation was carried out at least 2 wk before experimentation as previously described (27). All animals were OVX at least 1 month beforehand to eliminate cyclic alterations in the secretion of gonadal steroids. Lean animals were subjected to dietary restriction over a period of 6–10 months as described previously (24). Briefly, they were fed 500 g of pasture hay/d supplemented with straw for bulk to achieve body condition scores of 2 (28). Animals of normal body weight were kept on pasture, with hay supplementation for maintenance. The animals were weighed monthly and adjustments in food intake were made so that target weights of approximately 35 kg (lean) and 55 kg (normal) were attained. No animal in the lean group was excluded from feeding by dominant flock mates. When in single pens, lean animals were fed 500 g of Lucerne chaff per day and normal animals had *ad libitum* access to food. All experiments were carried out in the breeding season. All animal procedures were conducted with prior institutional ethical approval of the Animal Experimentation Ethics Committee of Monash University fulfilling the requirements of the Australian Prevention of Cruelty to Animals Act 1986 and the National Health and Medical Research Council/Commonwealth Scientific and Industrial Research Organization/Australian Animal Commission Code of Practice for the Care and Use of Animals for Scientific Purposes. Where possible, reduction and refinement was exercised using brain sections from animals that had already been treated for an earlier study (23).

Experimental design

Experiment 1: effect of body weight and leptin treatment on Kiss1 gene expression

Lean (33 ± 2.9 kg) OVX ewes (five per group) received IIIV infusions of either 4 μg/h human recombinant leptin (12) or artificial cerebrospinal fluid (aCSF; 150 mM NaCl, 1.2 mM CaCl, 1 mM MgCl, and 2.8 mM KCl) and normal (n = 4) weight ewes received aCSF as vehicle at a rate of 55 μl/h for 3 d using MS16A Grasby minipumps (Graseby Medical Ltd., Gold Coast, Australia). Blood samples (5 ml) were collected every 10 min for 6 h before infusion and for the final 6 h of the 72-h infusion period. Plasma was harvested and stored at −20 C until assayed for LH. At the end of the infusion period, animals were euthanized by overdose of 20 ml sodium pentobarbital (Lethabarb; Virbac, Peakhurst, New South Wales, Australia) iv, and the heads were removed and perfused through both carotid arteries with 2 liters normal saline containing heparin (12.5 U/ml) followed by 3 liters of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), the final liter containing 20% sucrose. Brains were removed, and the hypothalamus and POA were dissected as blocks and postfixed at 4 C in fixative containing 30% sucrose for 7 d. Cryostat sections were cut in the coronal plane (20 μm), collected into cryoprotectant with 2% paraformaldehyde and stored at −20 C until *Kiss1* expression was quantified by *in situ* hybridization (*vide infra*). These animals had been used in a previous experiment (23).

Experiment 2: expression of Ob-Rb in Kiss1 cells of the ARC and POA

Because leptin was found to regulate *Kiss1* mRNA expression in both the ARC and POA, we determined whether these cells

express the signaling leptin receptor, Ob-Rb. The brains of four OVX ewes of normal weight were perfused as above, and the hypothalami and POA were dissected as blocks and stored in ribonuclease-free 0.1 M PBS until cryostat sections were cut in the coronal plane (20 μm) and collected into cryoprotectant. The sections were stored at −20 C before immunohistochemical staining, laser capture, and real-time PCR (*vide infra*).

Experiment 3: reciprocal connections between kisspeptin cells and POMC/NPY cells in the ARC

OVX and gonad-intact ewes of normal body weight (in the luteal phase of the estrous cycle; n = 4/group) were euthanized, and the brains were perfused as above. Cryostat sections were cut in the coronal plane (40 μm), collected into cryoprotectant, and stored at −20 C for later immunohistochemical analysis of the connectivity between kisspeptin cells and the POMC and NPY cells of the ARC (*vide infra*).

Experiment 4: kisspeptin regulates POMC and NPY gene expression

OVX ewes (four per group) of normal body weight received IIIV infusion of 5 μg/h of kisspeptin (Phoenix Pharmaceuticals, Burlingame, CA) or aCSF at an infusion rate of 100 μl/h for 20 h after being fitted with intracerebroventricular infusion cannulae (SILASTIC brand tubing, Dow Corning, Midland, MI; inner diameter 1.02 mm, outer diameter 2.16 mm) in the lateral ventricle (23) connected to 5-ml plastic syringes driven by Graseby MS16A infusion pumps (Smith Medical Australasia Pty. Ltd., Gold Coast, Queensland, Australia). Blood samples were collected every half-hour for 1 h before and after the commencement of the infusion to monitor the LH response to kisspeptin. Plasma was harvested and stored at −20 C until assayed for an acute LH response. At the end of the 20-h infusion period, animals were euthanized and brains were perfused and sectioned as above to measure *POMC* and *NPY* gene expression by *in situ* hybridization (*vide infra*).

In situ hybridization

This was performed using ^{35}S-deoxyuridine 5-triphosphate-labeled riboprobes according to a described protocol (29, 30). The cDNA and plasmid inserts used were a 375-base ovine *Kiss1* sequence (31), a 400-base ovine *POMC* sequence (32), and a 511-base rat *NPY* sequence donated by Dr. Steven Sabol (National Heart, Blood, and Lung Institute, Bethesda, MD) (33). The amplification and linearization of plasmid DNA were performed using standard techniques (34). For analysis of ARC *Kiss1*, POMC, and NPY expression, three sections from each ewe were taken to represent rostral, middle, and caudal regions of the ARC. Three to five sections through the POA were chosen for *Kiss1* expression analysis in the dorsolateral region. All cRNA probes were synthesized using a Gemini System II kit (Promega, Annandale, New South Wales, Australia). After hybridization, the slides were dipped in Ilford K5 photographic emulsion (Ilford Imaging, Melbourne, Australia) and kept at 4 C in the dark and developed after 9 d (*Kiss1* in ARC), 12 d (*Kiss1* POA), 7 d (POMC), or 11 d (NPY). Sections were then counterstained with 1% cresyl violet, dehydrated, and coverslipped using DePex plastic resin antifade medium (Dako, Botany Bay, New South Wales, Australia). Image analysis was carried out using coded slides and the operator was blind to the treatments. Cells were counted when silver grain density was greater than 5 times background and there was a clearly discernible nucleus. Computer-assisted grain counting was performed under bright-light field at ×40 using a Fuji HC-2000 high-resolution digital camera and Analytical Imaging system 3.0 software (Image Pro Plus; Media Cybernetics, Silver Spring, MD), as previously described (35). The number of silver grains per cell for ARC *Kiss1*, *POMC*, and *NPY* mRNA was estimated in 40 cells per section in the ARC. In the POA *Kiss1* mRNA, silver grains/per cell was estimated in 20 cells per section or all cells if less than 20 cells were visible on the section.

Laser capture microdissection and real-time PCR for the determination of Ob-Rb on Kiss1 cells of the ARC and POA

Middle ARC and POA sections (n = 4) were selected from the tissues of sheep in experiment 2, and kisspeptin cells were identified in free-floating sections by ribonuclease-free immunohistochemistry. Briefly, sections were first washed in 0.1 M PBS and preincubated in proteinase K digestion for 5 min at 37 C. After washing sections again in 0.1 M PBS, sections were blocked (0.1 M PBS/1%BSA/0.3% Triton X-100) and kisspeptin cells were identified using a polyclonal rabbit antibody against mouse kisspeptin-10 (gift from Dr. A. Caraty, INRA, Nouzïlly, France) (36) at a dilution of 1:2000 in 0.1 M PBS/1% BSA/0.3% Triton X-100/0.1% NaN_3 at 24 h at room temperature. This antibody has been validated for use in ovine brain tissues (37). Sections were then rinsed in 0.1 M PBS and incubated for 30 min in goat antirabbit Alexa 488 secondary antibody (1:500; Molecular Probes, Eugene, OR) diluted in 0.1 M PBS. After a final wash, the sections were mounted onto membrane slides and allowed to dry overnight. Single cells were then isolated using the PALM MicroLaser microdissection system (P.A.L.M. MicroLaser Technologies AG, Burnried, Germany). Kisspeptin cells were dissected off the slide with a single defocused laser pulse and catapulted directly into the cap of microfuge tubes containing 40 μl of RTL buffer guanidine thiocyanate buffer from RNeasy microkit (QIAGEN, Hilden, Germany). Cells were collected as four groups of single cells per animal. Total RNA was extracted from the laser-captured cells using RNeasy microkit (QIAGEN) and reverse transcribed using AffinityScript QPCR cDNA synthesis kit (Stratagene, La Jolla, CA). To correct for variation in reverse transcriptase efficiency between samples, each RNA sample was spiked with a known amount of Alien RNA (Stratagene).

Nested primers for ovine *Ob-Rb* (outer primers 5′ aca gaa tca gcg acc ttt gg, 3′ gat tta gag ctg ccg ac gag; inner primers 5′ agt tga gat ggt gcc aac aa 3′ cag cct cag aga act cag ca; accession no. U62124) were used to enhance transcript detection and measurement. The initial PCR using outer primers and one tenth of the laser captured cell reverse transcriptase cDNA was set up for 95 C for 5 min, 14 cycles at 95 C for 1 min, 50 C for 1.30 min, 72 C for 1 min, and one cycle at 72 C for 5 min in a 25-μl reaction volume using GoTaq DNA polymerase (Promega, Madison, WI). Quantitative real-time PCR was performed on the Eppendorf Realplex (4) PCR (Eppendorf, Hamburg, Germany) machine using a reaction volume of 20 μl containing the inner primers and 2 μl of the first-round PCR. The PCR conditions were 95 C for 10 min followed by 55 cycles at 95 C for 15 sec, 56 C for 1 min, and 72 C for 30 sec using Brilliant SYBR Green QRT-PCR master mix (Stratagene). PCR conditions for the Alien RNA

(Stratagene) were 95 C for 10 min followed by 50 cycles at 95 C for 15 sec, 60 C for 45 sec, and 72 C for 30 sec.

Laser capture of single cells may include material above or below the cell in the sample. Three measures were taken to account for the possibility of contaminating material. First, we used sections of 20 μm, whereas the size of the kisspeptin cells is 10–15 μm (36). We sampled a number of single cells on the basis that repeated expression of the *Ob-Rb* gene in multiple cells would increase confidence that the cells of interest do indeed express the receptor. Then we conducted PCR of the same samples for gonadotropin-inhibiting hormone receptor (*GPR147*; outer primers 5′ gcg aga atg gaa gtg atg ct, 3′ ggc cag gtt gag gat aaa ca; inner primers 5′ tgc aaa cct cac ctt ctc ct, 3′ tga agc aga cca gcg tat tg; accession no. XM_61290). Ubiquitous expression of all receptors would indicate that our samples are nonspecific, whereas intermittent expression or low expression of other receptors would again increase confidence that there is consistent expression of the *Ob-Rb* in kisspeptin cells.

For the genes of interest, purified DNA of known concentration was used as the assay standard. In the initial optimization of the primer set, PCR products were separated by agarose gel electrophoresis, purified, and sequenced to confirm their identity. The estimated mRNA concentrations were determined relative to the standard preparation (concentration determined by Nanophotometer) using the Realplex[4] computer software. In the case of the Alien RNA (Stratagene), a separate reverse transcriptase was set up alongside the sample reverse transcriptase reactions containing a known amount of RNA, which was serially diluted and used as standard during the Alien RNA quantitative PCR run. *Ob-Rb* mRNA expression was corrected relative to the amount of Alien RNA measured in each sample.

Immunohistochemistry

Anatomically matching sections representing rostral, middle, and caudal ARC were selected from OVX and ovary intact ewes (n = 4/group) of experiment 3 and were mounted onto superfrost slides and dried overnight. POMC-containing cells were identified using a γMSH antibody (Antibodies Australia, Melbourne, Australia) with specificity as previously described (37). Preabsorption with 0.5 mg/ml of the original peptide abolished all staining in the ovine ARC (data not shown). To detect NPY, a monoclonal mouse antibody against NPY (1:4000, 72 h at 4 C; courtesy of E. Grouzmann, University Hospital, Lausanne, Switzerland) was used. Anti-guinea pig and antimouse secondary antibodies conjugated to Alexa 488 (1:500; Molecular Probes) were used as detection methods for γMSH and NPY, respectively. After staining for γMSH or NPY, the sections were then labeled for kisspeptin (as above). Sections were counterstained with 0.3% Sudan Black and coverslipped using DePex plastic resin antifade medium (Dako). Putative contacts of kisspeptin fibers on all detectable NPY and POMC neurons, and the reciprocal connections, were examined with a Zeiss Apotome microscope (Carl Zeiss, Inc., North Ryde, Sydney, Australia). Z-stacks of optical sections (1 μm; magnification, ×126) were captured through kisspeptin, NPY, and POMC-immunoreactive neurons. Putative contacts were defined as apposition of terminals with soma or proximal dendrites when there was no pixilation between the two objects. Using the Apotome system, Z-stacks were rotated to confirm the lack of pixilation between the objects, when viewed in different planes. This method has been reported previously (38).

RIAs

Plasma LH was measured by assaying samples in duplicate at 100 μl following the method of Lee *et al.* (39). Ovine standards were NIH-oLH-S18 and ovine antiserum (NIDDK-anti-oLH-I) was used with ^{125}I-NIDDK-AFD-9598B as tracer. The average sensitivity of the assays was 0.2 ng/ml and the interassay coefficient of variation was less than 15%. The intraassay coefficient of variation was less than 9%.

Statistics

Data are presented as means (±SEM). Hormone and leptin receptor data were analyzed by repeated-measures ANOVA, with least significant differences as a *post hoc* test. Measures of mRNA levels and close appositions were analyzed by one-way independent-measures ANOVA, with least significant differences *post hoc* comparison of normal weight, lean, and lean + leptin-treated animals. The percentage of cells with close appositions was examined using arc-sine transformed data.

Results

Experiment 1: effect of leptin on *Kiss1* expression

The plasma LH responses to leptin or vehicle in these animals has been reported previously (23). In summary, vehicle infusion had no effect on mean plasma LH levels, but leptin infusion increased mean LH concentrations ($P < 0.01$), LH pulse amplitude ($P < 0.01$), and LH pre-

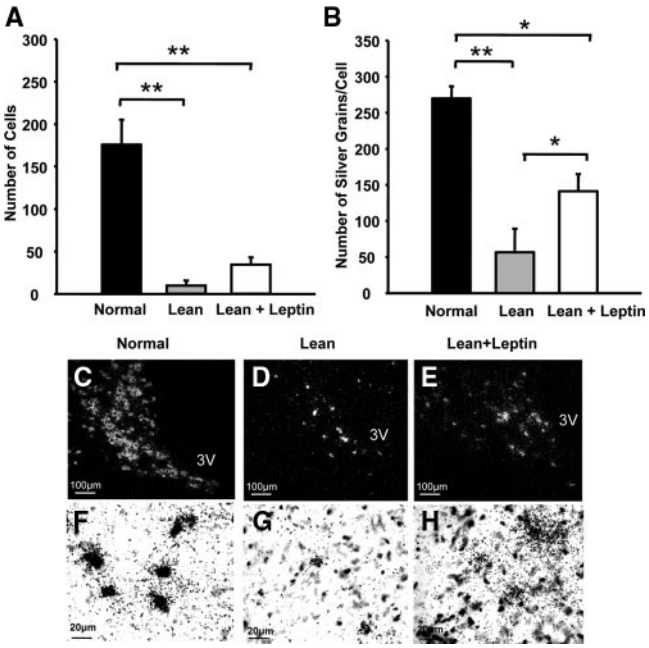

FIG. 1. *Kiss1* mRNA expression in the ARC of normal weight, lean, and lean + leptin-treated OVX ewes, as determined by *in situ* hybridization expressed as number of detectable *Kiss1* cells (A) or silver grains per cell (B). Dark-field photomicrographs of representative ARC sections from normal weight (C), lean (D), and lean + leptin-treated (E) OVX ewes at ×10 magnification. Bright-field photomicrographs of representative *Kiss1* cells in the ARC of normal-weight (F), lean (G), and lean + leptin-treated (H) OVX ewes at ×40 magnification. Data are means ± SEM. *, $P < 0.05$; **, $P < 0.01$.

pulse nadir ($P < 0.01$) in lean hypogonadotropic OVX ewes.

Kiss1 gene expression in the ARC was significantly ($P < 0.01$; cell number and silver grains per cell) lower in lean hypogonadotropic OVX ewes than ewes of normal body weight. Leptin treatment partially restored *Kiss1* mRNA toward normal by increasing the level of expression (silver grains per cell; $P < 0.05$; Fig. 1).

Kiss1 gene expression in the POA was significantly ($P < 0.05$) reduced in lean hypogonadotropic OVX ewes, in terms of the number of detectable cells and the level of expression per cell ($P < 0.01$) compared with ewes of normal body weight. Leptin treatment of lean hypogonadotropic OVX ewes partially restored the level of *Kiss1* mRNA expression per cell ($P < 0.05$) but not the number of detectable cells (Fig. 2).

Experiment 2: expression of *Ob-Rb* and *GPR147* in kisspeptin neurons of the ARC and POA

All single cells in the ARC and POA were seen to express *Ob-Rb*. When corrected for Alien RNA (Stratagene), mean relative *Ob-Rb* mRNA levels for single kisspeptin cells (four per animal) were 0.36 ± 0.15 and 0.17 ± 0.07 in the ARC and POA, respectively. *GPR147* was expressed in only two of 16 single cells captured in the ARC (four per animal) and four of 16 cells in the POA (four per animal). When corrected for Alien RNA (Stratagene), relative *GPR147* mRNA levels in single kisspeptin cells (in which *GPR147* was detected) ranged from 1.4×10^{-7} to 1.6×10^{-6} and 1.8×10^{-7} to 1.4×10^{-6} in the ARC and POA, respectively.

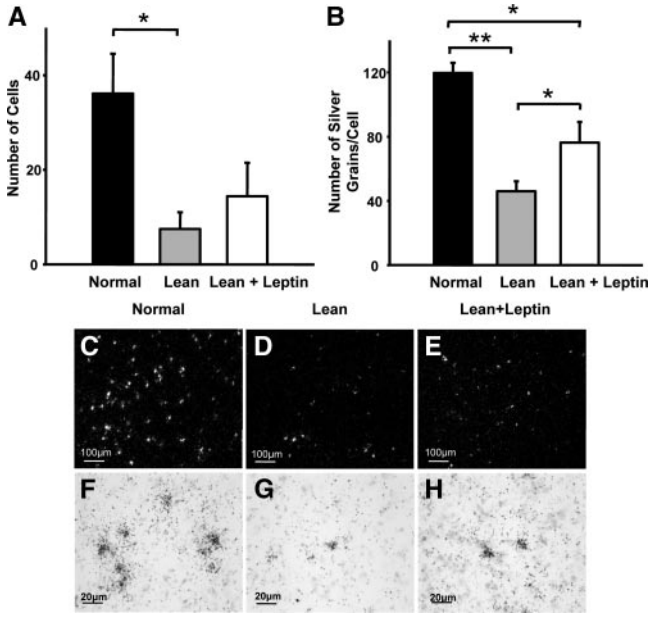

FIG. 2. *Kiss1* mRNA expression in the POA of normal-weight, lean, and lean + leptin-treated OVX ewes, as determined by *in situ* hybridization expressed as number of detectable *Kiss1* cells (A) or silver grains per cell (B). Dark-field photomicrographs of representative POA sections from normal-weight (C), lean (D), and lean + leptin-treated (E) OVX ewes at ×10 magnification. Bright-field photomicrographs of representative *Kiss1* cells in the POA of normal-weight (F), lean (G), and lean + leptin-treated (H) OVX ewes at ×40 magnification. Data are means ± SEM. *, $P < 0.05$; **, $P < 0.01$.

Experiment 3: reciprocal connections between kisspeptin and POMC/NPY cells

Kisspeptin fibers were seen in close apposition with 7.8 ± 2.5 and 7.1 ± 1.1% of NPY cells in intact and OVX ewes, respectively (Fig. 3). Close apposition of kisspeptin fibers was also with 22.9 ± 4.6 and 18.1 ± 1.8% of POMC cell bodies (intact and OVX ewes, respectively). Kisspeptin fibers were observed to be in close contact with POMC cell bodies more frequently that with NPY cell bodies in intact ($P < 0.05$) and OVX ewes ($P < 0.01$) (Fig. 3G).

NPY fibers were seen in close apposition with 13.9 ± 1.3 and 29.1 ± 3.1% of kisspeptin cells in intact and OVX ewes, respectively (Fig. 3, E, F, and H). POMC fibers were seen in close apposition with 44.1 ± 4.9 and 32.3 ± 6.9% kisspeptin cell bodies (intact and OVX ewes, respectively). POMC fibers came into contact with kisspeptin cells more frequently than NPY cell bodies in OVX ewes ($P < 0.01$). NPY fibers made close appositions with kisspeptin cells more frequently in OVX ewes than intact ewes ($P < 0.01$; Fig. 3H).

Experiment 4: effect of kisspeptin treatment on POMC and NPY gene expression and LH concentration

IIIV infusion of kisspeptin to OVX ewes increased ($P < 0.01$) *NPY* mRNA expression in the ARC in terms of the number of detectable cells and the level of expression per cell (Fig. 4, A–F). Kisspeptin reduced *POMC* mRNA expression in the ARC by reducing both the number of detectable cells ($P < 0.05$) and the level of expression per cell ($P < 0.01$ *vs.* vehicle treated controls; Fig. 4, G and H). LH concentration in these OVX ewes elevated after kisspeptin treatment (before infusion, 2.45 ± 0.84 ng/ml; after infusion, 2.73 ± 0.48 ng/ml; $P = 0.10$).

Discussion

The results of this study provide strong evidence that kisspeptin cells are regulated by leptin. This is most likely to be a direct effect on these cells in both the ARC and POA because both populations express Ob-Rb. Moreover, we present evidence of complex reciprocal relationships between kisspeptin cells and POMC/NPY cells in the ARC, which may synchronize the regulation of systems within the brain that modulate metabolic function as well as re-

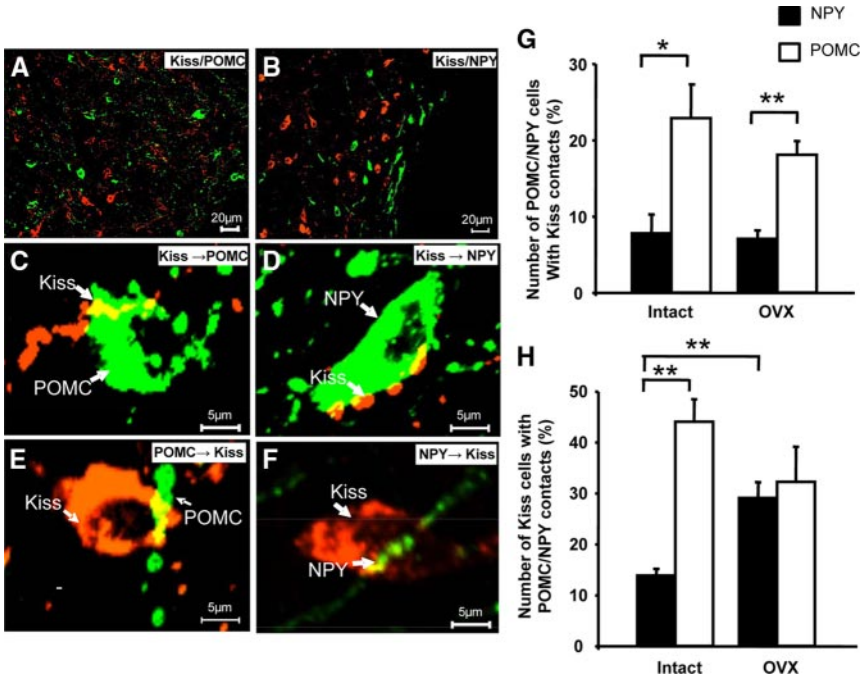

FIG. 3. Z-stack apotome analysis shows that kisspeptin fibers come into close contact with POMC and NPY cells, as do POMC and NPY fibers with kisspeptin cells. Low-power fluorescent photomicrograph indicating POMC (*green*) and kisspeptin (*red*) staining in the arcuate nucleus (A). B, NPY (*green*) and kisspeptin (*red*) in the ARC at low power). Digitally magnified image of a kisspeptin fiber coming in close apposition with a POMC cell (C) and NPY cell (D) is shown. Digitally magnified image of a POMC (E) and NPY (F) fiber coming in close contact with a kisspeptin cells is also shown. G, Average percentage of kisspeptin fibers contacting NPY and POMC cells in the ARC of intact and OVX ewes. H, Average percentage of POMC and NPY fibers contacting kisspeptin cells in the ARC of intact and OVX ewes. Data are means ± SEM. *, $P < 0.05$; **, $P < 0.01$.

production. We substantiate the notion that kisspeptin regulates POMC and NPY cells by altering their gene expression, allowing a means by which kisspeptin cells that are highly responsive to estradiol-17β might modulate appetite-regulating systems (NPY and POMC cells). It is possible that this is affected via direct transmission between the different cell types based on our histochemical data, but further definitive studies are required to confirm this.

Our data show a reduction in *Kiss1* gene expression in OVX lean sheep, which is partially restored by leptin. These findings confirm and extend previous data from work in both rats and mice (7, 9, 40). Gonadectomizing animals to remove sex steroids up-regulates *Kiss1* mRNA in the ARC (31) and could potentially minimize any inhibitory effect of negative energy balance on the expression of this gene. Nevertheless, we used OVX animals to obviate the confounding effect of variable levels of gonadal steroids and body weight/steroid feedback interactions. There are no papers of which we are aware that report the effect of leptin on *Kiss1* gene expression in gonad-intact animals, but a stimulatory effect would seem likely. In particular, the presence of sex steroids may influence the effect of leptin on these cells, which express high levels of estrogen receptor (ER)-α and progesterone receptor (31, 36). Gonadectomy also releases the reproductive system from the negative feedback constraint of gonadal steroids. In this setting, it is sometimes more difficult to discern the effects of regulatory peptides that are easily seen in the gonad-intact animal. This was the case in experiment 4 in which we did not observe a significant effect of kisspeptin on plasma LH levels in OVX ewes. This could be because of the infrequent sampling protocol that we used, dose of kisspeptin, or the fact that the free-running (elevated) pulsatile secretion of GnRH/LH masked any subliminal response.

In our model of the lean hypogonadotropic OVX ewe, which is in a stable metabolic condition (24) but has reduced energy stores in terms of adipose tissue, *Kiss1* mRNA expression is reduced in cells of both the ARC and POA. Previous studies concentrated on the kisspeptin cells of the ARC in the mouse (7), but our evidence of effect on the kisspeptin cells of the POA is important in terms of control of GnRH cells by kisspeptin. This is because there is no evidence of direct input to GnRH cells from cells of the ARC (41), nor do kisspeptin cells originating in the ARC project to the ventromedial POA (42) in which the majority of GnRH neurons are located (43). It is most likely that the high level of direct kisspeptin input to GnRH cells originates from the kisspeptin cells located in the POA (44). Regulation of GnRH cells by kisspeptin cells of the ARC may be effected by interneuronal pathways because cells in various regions of the POA and bed nucleus of the stria terminalis project directly to the GnRH cells (45).

The lowered level of *Kiss1* expression in lean animals may be at least one cause of the hypogonadotropic state because kisspeptin has a potent stimulatory effect on GnRH secretion (46, 47). This is supported by the demonstration that leptin treatment is able to partially restore the expression of *Kiss1* as well as pulsatile secretion of LH in hypogonadotropic lean animals (23). On the other hand, we have previously shown that *POMC* gene expression and melanocortin peptide levels are lowered in our lean ewe model, and leptin treatment restores the melanocortin system to normal, in concert with restoration of gonadotropic function (42). In this earlier paper, we presented evidence that the melanocortin system is a means by

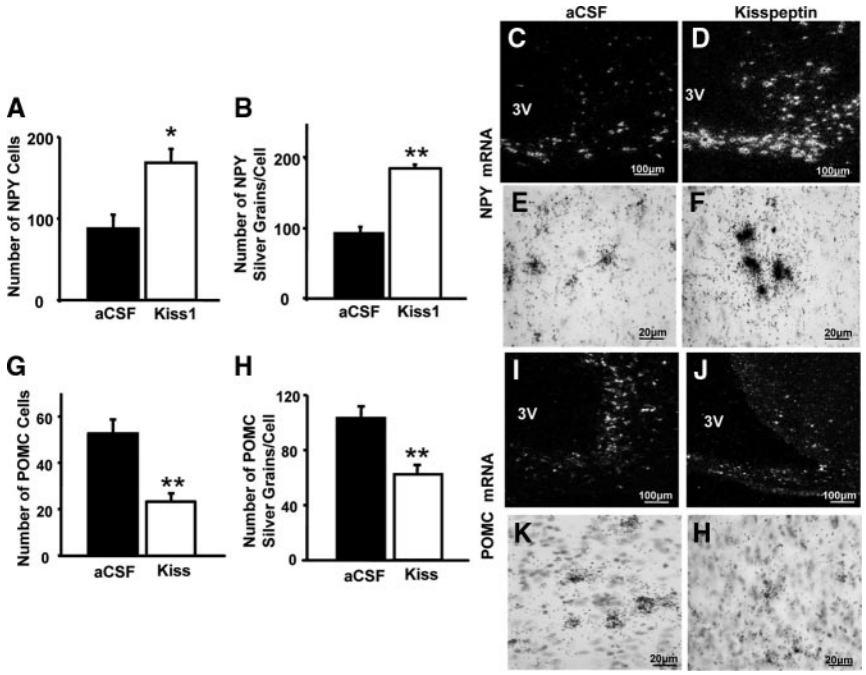

FIG. 4. Effect of IIIV of aCSF or kisspeptin (5 μg/h) for 20 h on *POMC* and *NPY* gene expression in the ARC of OVX ewes, expressed by the average number of cells per section and the number of silver grains per cell (A and B and G and H). Dark-field (×10) photomicrograph representations of aCSF treated (C and I) and kisspeptin treated (D and J) and bright-field (×40) of aCSF treated (E and K) and kisspeptin treated (F and H). *Solid bar*, aCSF treatment; *open bar*, kisspeptin treatment. Data are means ± SEM. *, $P < 0.05$; **, $P < 0.01$.

which leptin communicates with GnRH cells because GnRH cells do not express Ob-Rb (49–51). Given that an equivalent leptin dose fully restores *POMC* gene expression, in contrast to partially restoring *Kiss1* gene expression, it seems possible that the transmission of leptin signaling to GnRH cells is predominantly through the melanocortin system. Apart from kisspeptin originating from the subpopulation of cells in the POA acting directly on the GnRH cells, and the kisspeptin cells of the ARC acting indirectly, the ARC kisspeptin cells may modulate melanocortin transmission to GnRH cells as well as to regulate metabolic function. This possibility is outlined in Fig. 5, which provides a model of the integrated function of kisspeptin, NPY, and POMC neurons to regulate reproduction and metabolic function. It is possible that the kisspeptin cells provide a means by which sex steroids impact on neuroendocrine systems regulating food intake and energy expenditure.

A possible reason as to why we achieved only partial restoration of *Kiss1* gene expression in lean animals given leptin may be that other metabolic factors in addition to leptin also provide feedback information of metabolic status. These include insulin and glucose (52–54), ghrelin (55, 56), and fatty acids (57), which are known to act on the cells of the hypothalamus. Our lean OVX ewe model is characterized by not only a reduction in leptin levels but also lowered insulin concentrations (22, 55), although glucose and nonesterified fatty acid levels in plasma are similar to those in normal animals (24). In this model, leptin treatment does not change insulin levels (22), so this is unlikely to be a significant factor. Other recent studies show that leptin, but not insulin, stimulates *Kiss1* mRNA expression in mouse hypothalamic cell lines (5) and male diabetic rats (8). Earlier studies from this laboratory showed a trend toward an increase in *Ob-Rb* gene expression in the ARC of the lean OVX ewe when compared with normal-weight controls (55), so adequacy at this level is not likely to be a factor limiting leptin signaling. Ghrelin levels are reduced in the lean OVX ewe (55), but it is unknown whether this hormone affects *Kiss1* expression.

Corroborating earlier data obtained in mice (7), we found a high level of expression of Ob-Rb in ARC kisspeptin cells of the ewe. Furthermore, at least some of the cells previously observed to express Ob-Rb in the POA of the ovine brain (58) appear to be those that produce kisspeptin. There is little doubt that kisspeptin stimulates GnRH and LH secretion (1, 46, 47), and up-regulation of both ARC (59) and POA (60) subsets of these cells occurs in the late follicular phase of the estrous cycle, before the preovulatory surge in GnRH/LH secretion. In addition, an estradiol-17β stimulus that produces a surge in GnRH/LH secretion in the OVX ewe produces a robust fos response in cells of the ARC (61), consistent with earlier work showing that the region of the brain in which estrogen acts to cause the surge is within the ARC/ventromedial hypothalamus (62, 63). This is likely to be through at least one interneuronal chain because we have found that very few ARC kisspeptin cells project to the ventromedial POA (42), confirming anterograde tracing results (41). It is possible that the ARC kisspeptin cells do, however, project to the dorsolateral POA and the bed nucleus of the stria terminalis, from which there are direct connections to the GnRH cells (45).

The demonstrated presence of Ob-Rb expression in all of the laser-captured kisspeptin neurons of the ARC substantiates the notion that these cells respond to leptin. Furthermore, Ob-Rb expression in the kisspeptin cells of the POA provides a novel conduit for leptin regulation of GnRH/gonadotropin secretion. There was some heterogeneity in the level of expression Ob-Rb in individual la-

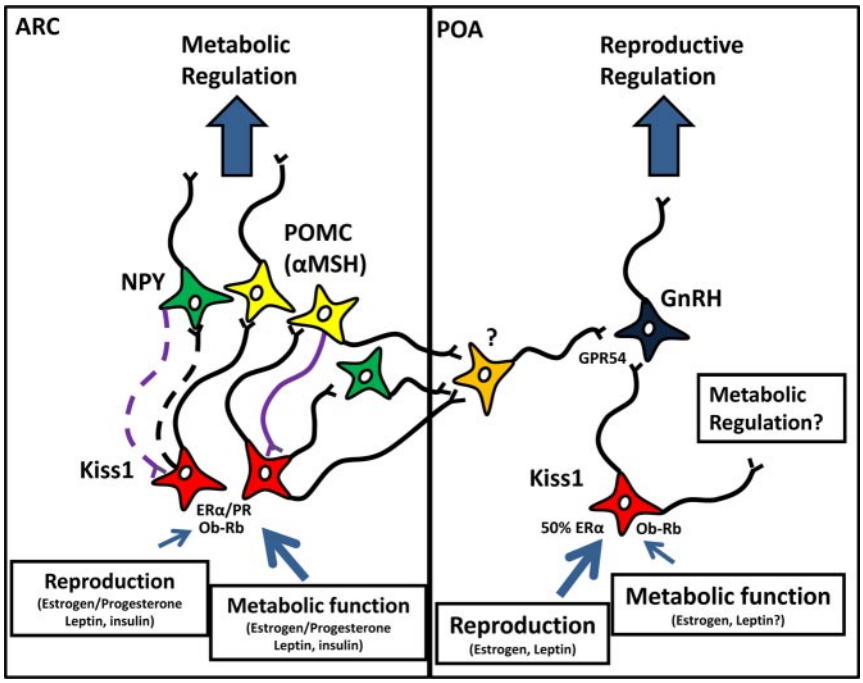

FIG. 5. Model of the integrated function of kisspeptin neurons to regulate reproduction and metabolic function. *ARC panel*, In the ARC, kisspeptin cells possess ERα, progesterone receptors (PRs), and leptin receptors (Ob-Rb). These cells may also possess other receptors that detect metabolic signals (insulin, ghrelin, *etc.*). By sensing leptin and sex steroid levels, the kisspeptin cells may relay information to NPY and POMC cells (*black lines*). NPY and POMC cells possess leptin receptors (and a subset possess ERα) and reciprocal connections (*purple*) provide possible regulation of the reproductive system through kisspeptin cells (*dashed lines* indicate a lower percentage of synaptic contacts compared with POMC/kisspeptin putative connections). It is also known that melanocortins stimulate the reproductive axis (23), and melanocortin cells provide synaptic input to kisspeptin cells in the ARC, so both melanocortin cells and kisspeptin cells may act in concert to control GnRH cells. Regulation of GnRH cells by kisspeptin and melanocortin cells of the ARC occurs via at least one interneuron (41, 42). In addition to controlling reproduction, the integrated kisspeptin/NPY/melanocortin system of the ARC may regulate food intake and energy expenditure. Thus, effects of leptin may be directly on NPY/POMC cells or indirectly via kisspeptin cells. Furthermore, the sex steroid effects to regulate food intake and energy expenditure may be relayed via kisspeptin cells, which express the relevant receptors at high level. *POA panel*, Kisspeptin cells express leptin receptors and 50% of these cells express ERs. The kisspeptin cells in the POA may provide direct input to GnRH cells (which posses GPR54) to regulate reproduction. By action on the kisspeptin cells in this region of the brain, estadiol-17β and leptin may control reproductive function. It is also known that cells in this region of the brain control homeostatic processes (48), so it cannot be ruled out that kisspeptin cells are important in this regard.

ser-captured cells, suggestive of a cell type with diverse physiological functions. This raises the possibility that some of these cells engage in control of metabolic function, whereas others perform a role in the regulation of reproduction. To gain confidence that our data from laser-captured cells represent true localization to these cells, we adopted various procedures. First, we minimized the thickness of the histological sections to 20 μm, whereas the size of the kisspeptin cells is 10–15 μm (36). Second, we conducted PCR for GPR147. If there were generalized expression of another receptor, this might reduce confidence that there is specific expression of Ob-Rb in the cells of interest. The data for the GPR147 showed that kisspeptin cells had a relatively lower level of expression than that for Ob-Rb, suggesting that our results for the latter are not of a nonspecific nature. Third, we examined a number of single cells in each animal on the basis that consistent high levels of expression in each cell would indicate a high possibility of expression being localized to the cells and not to surrounding tissues. Consistent high levels of expression were seen in each single cell that was examined.

We have presented evidence that NPY and POMC cells project to kisspeptin cells in the ARC of OVX and intact ewes. This higher input of POMC cells to kisspeptin may perhaps indicate that the melanocortin cells are fundamentally more instrumental in synchronizing effects of peripheral signaling agents on the metabolic and reproductive systems of the hypothalamus than are the NPY cells. In support of this histochemical data are the observation that kisspeptin treatment reduced POMC expression and increased NPY expression. Because almost 100% of ARC kisspeptin cells express ER-α (36) and progesterone receptor (31), but only a small subset of POMC (43) and NPY (64) cells express sex steroid receptors, the ARC kisspeptin neurons may allow significant (indirect) sex steroid regulation of NPY and POMC cells, which may be a mechanism to regulate metabolic systems as well as reproduction. The high level of Ob-Rb in kisspeptin cells may be a complementary pathway by which metabolic status is transmitted to the reproductive axis. If this nexus were part of an intricate feedback system to regulate the reproductive system, one might expect some degree of synaptic plasticity regarding the extent to which kisspeptin fibers contact POMC and NPY cells, but we did not find any differences between gonad-intact and OVX ewes. The novel finding that kisspeptin regulates POMC and NPY cells provides a means by which the well-known effects of sex steroids on food intake and energy expenditure (65–67) might be caused. In human females, food intake peaks in the luteal phase of the estrous cycle when estrogen is at its lowest and is at nadir during the periovulatory period when estrogen level reach peak concentrations (68). Recent research, however, implies that the kisspeptin system is not involved in the regulation of food intake. Acute kisspeptin treatment of lean animals at a dose ef-

fective to increase LH secretion does not alter food intake (7, 34), and GPR54 knockout mice display no obvious phenotype of metabolic disruption (69). These studies, however, investigated the effect of short-term kisspeptin treatment on food intake, and a more sustained infusion of kisspeptin may be needed. Such studies are in progress in our laboratory.

These results that kisspeptin reduces *POMC* gene expression may appear as somewhat perplexing, given the facilitatory effect of αMSH to stimulate GnRH neuron activity. One may then expect that an increase in ARC kisspeptin would reduce activation of GnRH neurons, acting in an opposing manner to the well-documented ability of kisspeptin to increase LH secretion (1–4). There are two possible explanations for this paradoxical finding. First, continuous infusion of kisspeptin has been shown to desensitize the stimulatory LH response in both monkeys (70) and sheep (46). It is therefore possible that the effect seen on *NPY* and *POMC* gene expression, which is observed at the end of a continuous 20-h kisspeptin infusion, is at a time when LH levels are low and kisspeptin may no longer be having a stimulatory effect on LH secretion. Kisspeptin may stimulate the melanocortin system and *POMC* mRNA under different physiological conditions. Future experiments may require the analysis of *POMC* and *NPY* mRNA and peptide after both acute and chronic infusion to completely resolve this matter. Alternatively, kisspeptin cells originating in the ARC may be more related to food intake/energy expenditure than reproduction. Indeed, gonadectomy increases food intake and reduces energy expenditure (65, 71), leading to increased adiposity. It is possible that sex steroid effects mediated through kisspeptin cells act to reduce food intake and increase energy expenditure, and because sex steroids hold kisspeptin levels down, removal of the sex steroids (by gonadectomy) with increased kisspeptin expression would then tend to reduce POMC and increase NPY, leading to increased food intake and reduced energy expenditure with a relatively greater degree of adiposity.

Lastly, we revealed that POMC and NPY cells project to kisspeptin cells in the ARC. This is important because these pathways may provide an indirect conduit to GnRH cells. With respect to melanocortin production by POMC cells and the stimulatory effect on the reproductive axis (42), an effect via kisspeptin cells is one possible conduit, in addition to the possible role of Orexin cells (42). Notably, more than 40% of kisspeptin cells also receive reciprocal input from melanocortin cells. This allows the melanocortin cells, 100% of which express Ob-Rb, to influence kisspeptin cells, which could further explain the leptin effect on the latter and may serve to amplify the effect of leptin on kisspeptin cells, which could then be transmitted to GnRH cells.

In summary, we demonstrate that leptin status regulates Ob-Rb expressing kisspeptin cells in the ARC and POA. Complex reciprocal connections between the ARC kisspeptin cells and the POMC cells provide a neuronal network through which metabolic signals and sex steroid signals may affect both cell types (summarized in Fig. 5); these in turn may regulate reproduction and metabolic function. The reciprocal connections between NPY and kisspeptin cells may be of lesser importance.

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References

1. **Irwig MS, Fraley GS, Smith JT, Acohido BV, Popa SM, Cunningham MJ, Gottsch ML, Clifton DK, Steiner RA** 2004 Kisspeptin activation of gonadotropin releasing hormone neurons and regulation of KiSS-1 mRNA in the male rat. Neuroendocrinology 80:264–272
2. **Smith JT, Clifton DK, Steiner RA** 2006 Regulation of the neuroendocrine reproductive axis by kisspeptin-GPR54 signaling. Reproduction 131:623–630
3. **Shahab M, Mastronardi C, Seminara SB, Crowley WF, Ojeda SR, Plant TM** 2005 Increased hypothalamic GPR54 signaling: a potential mechanism for initiation of puberty in primates. Proc Natl Acad Sci USA 102:2129–2134
4. **Dhillo WS, Chaudhri OB, Patterson M, Thompson EL, Murphy KG, Badman MK, McGowan BM, Amber V, Patel S, Ghatei MA, Bloom SR** 2005 Kisspeptin-54 stimulates the hypothalamic-pituitary gonadal axis in human males. J Clin Endocrinol Metab 90:6609–6615
5. **Luque RM, Kineman RD, Tena-Sempere M** 2007 Regulation of hypothalamic expression of KiSS-1 and GPR54 genes by metabolic factors: analyses using mouse models and a cell line. Endocrinology 148:4601–4611
6. **Brown RE, Imran SA, Ur E, Wilkinson M** 2008 KiSS-1 mRNA in adipose tissue is regulated by sex hormones and food intake. Mol Cell Endocrinol 281:64–72
7. **Smith JT, Acohido BV, Clifton DK, Steiner RA** 2006 KiSS-1 neurones are direct targets for leptin in the ob/ob mouse. J Neuroendocrinol 18:298–303
8. **Castellano JM, Navarro VM, Fernández-Fernández R, Roa J, Vigo E, Pineda R, Dieguez C, Aguilar E, Pinilla L, Tena-Sempere M** 2006 Expression of hypothalamic KiSS-1 system and rescue of defective gonadotropic responses by kisspeptin in streptozotocin-induced diabetic male rats. Diabetes 55:2602–2610
9. **Castellano JM, Navarro VM, Fernández-Fernández R, Nogueiras R, Tovar S, Roa J, Vazquez MJ, Vigo E, Casanueva FF, Aguilar E, Pinilla L, Dieguez C, Tena-Sempere M** 2005 Changes in hypothalamic KiSS-1 system and restoration of pubertal activation of the reproductive axis by kisspeptin in undernutrition. Endocrinology 146:3917–3925
10. **Chehab FF, Lim ME, Lu R** 1996 Correction of the sterility defect in

homozygous obese female mice by treatment with the human recombinant leptin. Nat Genet 12:318–320
11. **Barash IA, Cheung CC, Weigle DS, Ren H, Kabigting EB, Kuijper JL, Clifton DK, Steiner RA** 1996 Leptin is a metabolic signal to the reproductive system. Endocrinology 137:3144–3147
12. **Henry BA, Goding JW, Tilbrook AJ, Dunshea FR, Clarke IJ** 2001 Intracerebroventricular infusion of leptin elevates the secretion of luteinising hormone without affecting food intake in long-term food-restricted sheep, but increases growth hormone irrespective of bodyweight. J Endocrinol 168:67–77
13. **Crown A, Clifton DK, Steiner RA** 2007 Neuropeptide signaling in the integration of metabolism and reproduction. Neuroendocrinology 86:175–182
14. **Cone RD** 2005 Anatomy and regulation of the central melanocortin system. Nat Neurosci 8:571–578
15. **Clarke IJ, Backholer K, Tilbrook AJ** 2005 Y2 receptor-selective agonist delays the estrogen-induced luteinizing hormone surge in ovariectomized ewes, but y1-receptor-selective agonist stimulates voluntary food intake. Endocrinology 146:769–775
16. **Nijenhuis WA, Oosterom J, Adan RA** 2001 AgRP(83-132) acts as an inverse agonist on the human-melanocortin-4 receptor. Mol Endocrinol 15:164–171
17. **Schioth HB, Kakizaki Y, Kohsaka A, Suda T, Watanobe H** 2001 Agouti-related peptide prevents steroid-induced luteinizing hormone and prolactin surges in female rats. Neuroreport 12:687–690
18. **Fan W, Boston BA, Kesterson RA, Hruby VJ, Cone RD** 1997 Role of melanocortinergic neurons in feeding and the agouti obesity syndrome. Nature 385:165–168
19. **Watanobe H, Schiöth HB, Wikberg JE, Suda T** 1999 The melanocortin 4 receptor mediates leptin stimulation of luteinizing hormone and prolactin surges in steroid-primed ovariectomized rats. Biochem Biophys Res Commun 257:860–864
20. **Ahima RS, Hileman SM** 2000 Postnatal regulation of hypothalamic neuropeptide expression by leptin: implications for energy balance and body weight regulation. Regul Pept 92:1–7
21. **Wang Q, Bing C, Al-Barazanji K, Mossakowaska DE, Wang XM, McBay DL, Neville WA, Taddayon M, Pickavance L, Dryden S, Thomas ME, McHale MT, Gloyer IS, Wilson S, Buckingham R, Arch JR, Trayhurn P, Williams G** 1997 Interactions between leptin and hypothalamic neuropeptide Y neurons in the control of food intake and energy homeostasis in the rat. Diabetes 46:335–341
22. **Henry BA, Goding JW, Alexander WS, Tilbrook AJ, Canny BJ, Dunshea F, Rao A, Mansell A, Clarke IJ** 1999 Central administration of leptin to ovariectomized ewes inhibits food intake without affecting the secretion of hormones from the pituitary gland: evidence for a dissociation of effects on appetite and neuroendocrine function. Endocrinology 140:1175–1182
23. **Backholer K, Bowden M, Gamber K, Bjorbaek C, Iqbal J, Clarke IJ** 2010 Melanocortins mimic the effects of leptin to restore reproductive function in lean hypogonadotropic ewes. Neuroendocrinology 91:27–40
24. **Henry BA, Tilbrook AJ, Dunshea FR, Rao A, Blache D, Martin GB, Clarke IJ** 2000 Long-term alterations in adiposity affect the expression of melanin-concentrating hormone and enkephalin but not proopiomelanocortin in the hypothalamus of ovariectomized ewes. Endocrinology 141:1506–1514
25. **Korner J, Savontaus E, Chua Jr SC, Leibel RL, Wardlaw SL** 2001 Leptin regulation of Agrp and Npy mRNA in the rat hypothalamus. J Neuroendocrinol 13:959–966
26. **Thornton JE, Cheung CC, Clifton DK, Steiner RA** 1997 Regulation of hypothalamic proopiomelanocortin mRNA by leptin in ob/ob mice. Endocrinology 138:5063–5066
27. **Barker-Gibb ML, Scott CJ, Boublik JH, Clarke IJ** 1995 The role of neuropeptide Y (NPY) in the control of LH secretion in the ewe with respect to season, NPY receptor subtype and the site of action in the hypothalamus. J Endocrinol 147:565–579
28. **Russel AJF, Doney JM, Gunn RG** 1969 Subjective assessment of body fat in live sheep. J Agric Sci Technol 72:451–454
29. **Simmons DM AJ, Swanson LW** 1989 A complete protocol for *in situ* hybridization of messenger RNAs in brain and other tissues with radio-labelled single-stranded RNA probes. J Histotechnol 12:169–181
30. **Scott CJ, Pereira AM, Rawson JA, Simmons DM, Rossmanith WG, Ing NH, Clarke IJ** 2000 The distribution of progesterone receptor immunoreactivity and mRNA in the preoptic area and hypothalamus of the ewe: upregulation of progesterone receptor mRNA in the mediobasal hypothalamus by oestrogen. J Neuroendocrinol 12:565–575
31. **Smith JT, Clay CM, Caraty A, Clarke IJ** 2007 KiSS-1 messenger ribonucleic acid expression in the hypothalamus of the ewe is regulated by sex steroids and season. Endocrinology 148:1150–1157
32. **van de Pavert SA, Clarke IJ, Rao A, Vrana KE, Schwartz J** 1997 Effects of vasopressin and elimination of corticotropin-releasing hormone-target cells on pro-opiomelanocortin mRNA levels and adrenocorticotropin secretion in ovine anterior pituitary cells. J Endocrinol 154:139–147
33. **Estrada KM, Pompolo S, Morris MJ, Tilbrook AJ, Clarke IJ** 2003 Neuropeptide Y (NPY) delays the oestrogen-induced luteinizing hormone (LH) surge in the ovariectomized ewe: further evidence that NPY has a predominant negative effect on LH secretion in the ewe. J Neuroendocrinol 15:1011–1020
34. **Sambrook J, Fritsch E, Maniatis T** 1989 Molecular cloning: a laboratory manual. 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press
35. **Henry BA, Rao A, Ikenasio BA, Mountjoy KG, Tilbrook AJ, Clarke IJ** 2001 Differential expression of cocaine- and amphetamine-regulated transcript and agouti related-protein in chronically food-restricted sheep. Brain Res 918:40–50
36. **Franceschini I, Lomet D, Cateau M, Delsol G, Tillet Y, Caraty A** 2006 Kisspeptin immunoreactive cells of the ovine preoptic area and arcuate nucleus co-express estrogen receptor α. Neurosci Lett 401: 225–230
37. **Goodman RL, Lehman MN, Smith JT, Coolen LM, de Oliveira CV, Jafarzadehshirazi MR, Pereira A, Iqbal J, Caraty A, Ciofi P, Clarke IJ** 2007 Kisspeptin neurons in the arcuate nucleus of the ewe express both dynorphin A and neurokinin B. Endocrinology 148:5752–5760
38. **Qi Y, Oldfield BJ, Clarke IJ** 2009 Projections of RFamide-related peptide-3 neurons in the ovine hypothalamus, with special reference to regions regulating energy balance and reproduction. J Neuroendocrinol 21:690–697
39. **Lee VW, Cumming IA, de Kretser DM, Findlay JK, Hudson B, Keogh EJ** 1976 Regulation of gonadotrophin secretion in rams from birth to sexual maturity. I. Plasma LH, FSH and testosterone levels. J Reprod Fertil 46:1–6
40. **Xu J, Kirigiti MA, Grove KL, Smith MS** 2009 Regulation of food intake and gonadotropin-releasing hormone/luteinizing hormone during lactation: role of insulin and leptin. Endocrinology 150:4231–4240
41. **Pompolo S, Rawson JA, Clarke IJ** 2001 Projections from the arcuate/ventromedial region of the hypothalamus to the preoptic area and bed nucleus of stria terminalis in the brain of the ewe: lack of direct input to gonadotropin-releasing hormone neurons. Brain Res 904: 1–12
42. **Backholer K, Smith J, Clarke IJ** 2009 Melanocortins may stimulate reproduction by activating orexin neurons in the dorsomedial hypothalamus and kisspeptin neurons in the preoptic area of the ewe. Endocrinology 150:5488–5497
43. **Lehman MN, Karsch FJ** 1993 Do gonadotropin-releasing hormone, tyrosine hydroxylase-, and β-endorphin-immunoreactive neurons contain estrogen receptors? A double-label immunocytochemical study in the Suffolk ewe. Endocrinology 133:887–895
44. **Smith JT, Coolen LM, Kriegsfeld LJ, Sari IP, Jaafarzadehshirazi MR, Maltby M, Bateman K, Goodman RL, Tilbrook AJ, Ubuka T, Bentley GE, Clarke IJ, Lehman MN** 2008 Variation in kisspeptin and RFamide-related peptide (RFRP) expression and terminal con-

nections to gonadotropin-releasing hormone neurons in the brain: a novel medium for seasonal breeding in the sheep. Endocrinology 149:5770–5782

45. **Pompolo S, Ischenko O, Pereira A, Iqbal J, Clarke IJ** 2005 Evidence that projections from the bed nucleus of the stria terminalis and from the lateral and medial regions of the preoptic area provide input to gonadotropin releasing hormone (GNRH) neurons in the female sheep brain. Neuroscience 132:421–436
46. **Caraty A, Smith JT, Lomet D, Ben Saïd S, Morrissey A, Cognie J, Doughton B, Baril G, Briant C, Clarke IJ** 2007 Kisspeptin synchronizes preovulatory surges in cyclical ewes and causes ovulation in seasonally acyclic ewes. Endocrinology 148:5258–5267
47. **Thompson EL, Patterson M, Murphy KG, Smith KL, Dhillo WS, Todd JF, Ghatei MA, Bloom SR** 2004 Central and peripheral administration of kisspeptin-10 stimulates the hypothalamic-pituitary-gonadal axis. J Neuroendocrinol 16:850–858
48. **Dagnault A, Richard D** 1997 Involvement of the medial preoptic area in the anorectic action of estrogens. Am J Physiol 272:R311–R317
49. **Hâkansson ML, Brown H, Ghilardi N, Skoda RC, Meister B** 1998 Leptin receptor immunoreactivity in chemically defined target neurons of the hypothalamus. J Neurosci 18:559–572
50. **Quennell JH, Mulligan AC, Tups A, Liu X, Phipps SJ, Kemp CJ, Herbison AE, Grattan DR, Anderson GM** 2009 Leptin indirectly regulates GnRH neuronal function. Endocrinology 150:2805–2812
51. **Finn PD, Cunningham MJ, Pau KY, Spies HG, Clifton DK, Steiner RA** 1998 The stimulatory effect of leptin on the neuroendocrine reproductive axis of the monkey. Endocrinology 139:4652–4662
52. **Szymanski LA, Schneider JE, Friedman MI, Ji H, Kurose Y, Blache D, Rao A, Dunshea FR, Clarke IJ** 2007 Changes in insulin, glucose and ketone bodies, but not leptin or body fat content precede restoration of luteinising hormone secretion in ewes. J Neuroendocrinol 19:449–460
53. **Miller DW, Blache D, Boukhliq R, Curlewis JD, Martin GB** 1998 Central metabolic messengers and the effects of nutrition on gonadotrophin secretion in sheep. J Reprod Fertil 112:347–356
54. **Parton LE, Ye CP, Coppari R, Enriori PJ, Choi B, Zhang CY, Xu C, Vianna CR, Balthasar N, Lee CE, Elmquist JK, Cowley MA, Lowell BB** 2007 Glucose sensing by POMC neurons regulates glucose homeostasis and is impaired in obesity. Nature 449:228–232
55. **Kurose Y, Iqbal J, Rao A, Murata Y, Hasegawa Y, Terashima Y, Kojima M, Kangawa K, Clarke IJ** 2005 Changes in expression of the genes for the leptin receptor and the growth hormone-releasing peptide/ghrelin receptor in the hypothalamic arcuate nucleus with long-term manipulation of adiposity by dietary means. J Neuroendocrinol 17:331–340
56. **Wren AM, Small CJ, Abbott CR, Dhillo WS, Seal LJ, Cohen MA, Batterham RL, Taheri S, Stanley SA, Ghatei MA, Bloom SR** 2001 Ghrelin causes hyperphagia and obesity in rats. Diabetes 50:2540–2547
57. **Tapsell L, Batterham M, Huang XF, Tan SY, Teuss G, Charlton K, Oshea J, Warensjo E** 30 June 2009 Short term effects of energy restriction and dietary fat sub-type on weight loss and disease risk factors. Nutr Metab Cardiovasc Dis 10.1016/j.numecd.2009.04.007
58. **Williams LM, Adam CL, Mercer JG, Moar KM, Slater D, Hunter L, Findlay PA, Hoggard N** 1999 Leptin receptor and neuropeptide Y gene expression in the sheep brain. J Neuroendocrinol 11:165–169
59. **Estrada KM, Clay CM, Pompolo S, Smith JT, Clarke IJ** 2006 Elevated KiSS-1 expression in the arcuate nucleus prior to the cyclic preovulatory gonadotrophin-releasing hormone/lutenising hormone surge in the ewe suggests a stimulatory role for kisspeptin in oestrogen-positive feedback. J Neuroendocrinol 18:806–809
60. **Smith JT, Li Q, Pereira A, Clarke IJ** 2009 Kisspeptin neurons in the ovine arcuate nucleus and preoptic area are involved in the preovulatory luteinizing hormone surge. Endocrinology 150:5530–5538
61. **Smith JT, Popa SM, Clifton DK, Hoffman GE, Steiner RA** 2006 Kiss1 neurons in the forebrain as central processors for generating the preovulatory luteinizing hormone surge. J Neurosci 26:6687–6694
62. **Blache D, Fabre-Nys CJ, Venier G** 1991 Ventromedial hypothalamus as a target for oestradiol action on proceptivity, receptivity and luteinizing hormone surge of the ewe. Brain Res 546:241–249
63. **Caraty A, Fabre-Nys C, Delaleu B, Locatelli A, Bruneau G, Karsch FJ, Herbison A** 1998 Evidence that the mediobasal hypothalamus is the primary site of action of estradiol in inducing the preovulatory gonadotropin releasing hormone surge in the ewe. Endocrinology 139:1752–1760
64. **Skinner DC, Herbison AE** 1997 Effects of photoperiod on estrogen receptor, tyrosine hydroxylase, neuropeptide Y, and β-endorphin immunoreactivity in the ewe hypothalamus. Endocrinology 138:2585–2595
65. **Asarian L, Geary N** 2002 Cyclic estradiol treatment normalizes body weight and restores physiological patterns of spontaneous feeding and sexual receptivity in ovariectomized rats. Horm Behav 42:461–471
66. **Parker GC, McKee ME, Bishop C, Coscina DV** 2001 Whole-body metabolism varies across the estrous cycle in Sprague-Dawley rats. Physiol Behav 74:399–403
67. **Shi H, Clegg DJ** 2009 Sex differences in the regulation of body weight. Physiol Behav 97:199–204
68. **Buffenstein R, Poppitt SD, McDevitt RM, Prentice AM** 1995 Food intake and the menstrual cycle: a retrospective analysis, with implications for appetite research. Physiol Behav 58:1067–1077
69. **de Roux N, Genin E, Carel JC, Matsuda F, Chaussain JL, Milgrom E** 2003 Hypogonadotropic hypogonadism due to loss of function of the *Kiss1*-derived peptide receptor GPR54. Proc Natl Acad Sci USA 100:10972–10976
70. **Seminara SB, Dipietro MJ, Ramaswamy S, Crowley Jr WF, Plant TM** 2006 Continuous human metastin 45–54 infusion desensitizes G protein-coupled receptor 54-induced gonadotropin-releasing hormone release monitored indirectly in the juvenile male Rhesus monkey (*Macaca mulatta*): a finding with therapeutic implications. Endocrinology 147:2122–2126
71. **Rogers NH, Perfield 2nd JW, Strissel KJ, Obin MS, Greenberg AS** 2009 Reduced energy expenditure and increased inflammation are early events in the development of ovariectomy-induced obesity. Endocrinology 150:2161–2168