Melanocortins May Stimulate Reproduction by Activating Orexin Neurons in the Dorsomedial Hypothalamus and Kisspeptin Neurons in the Preoptic Area of the Ewe

Kathryn Backholer, Jeremy Smith, and Iain J. Clarke

Department of Physiology, Monash University, Clayton, Victoria 3800, Australia

To further test the hypothesis that melanocortins stimulate the reproductive axis, we treated ewes with melanocortin agonist (MTII) in the luteal phase of the estrous cycle and during seasonal anestrus. Lateral ventricular infusion of MTII (10 μg/h) during the luteal phase increased LH secretion. Retrograde neuronal tracing in the brain showed few proopiomelanocortin or kisspeptin cells in the arcuate nucleus, but more than 70% of kisspeptin cells in the dorsolateral preoptic area (POA), projecting to the ventromedial POA in which GnRH cells are located. MTII infusion (20 h) was repeated in luteal phase ewes and brains were harvested to measure gene expression of prepro-orexin and kisspeptin. Expression of orexin in the dorsomedial hypothalamus and kisspeptin in the POA was up-regulated by MTII treatment and Kiss1 in the arcuate nucleus was down-regulated. Seasonally anestrous ewes were progesterone primed and then treated (lateral ventricular) with MTII (10 μg/h) or vehicle for 30 h, and blood samples were collected every 2 h from 4 h before infusion until 6 h afterward to monitor acute response in terms of LH levels. A rise in basal LH levels was seen, but samples collected around the time of the predicted LH surge did not indicate that an ovulatory event occurred. We conclude that melanocortins are positive regulators of the reproductive neuroendocrine system, but treatment with melanocortins does not fully overcome seasonal acyclicity. The stimulatory effect of melanocortin in the luteal phase of the estrous cycle may be via the activation of kisspeptin cells in the POA and/or orexin cells in the dorsomedial hypothalamus. **(*Endocrinology* 150: 5488–5497, 2009)**

Posttranslational processing of the proopiomelanocortin (POMC) gene results in the formation of endorphin and melanocortin products in the hypothalamus (1). Whereas POMC mRNA expression in the arcuate nucleus (ARC) appears to be unchanged across the estrous cycle in the ewe (2), sex steroid effects on posttranslational processing of POMC remains to be determined and differential processing of the POMC precursor peptide could occur. In the rat, POMC mRNA expression is reduced at the time of the preovulatory LH surge (3, 4), and treatment of ovariectomized (OVX) rats with estrogen reduces POMC mRNA (5–7); progesterone treatment is able to attenuate or reverse this effect (3, 6). On the other hand, others have shown that a reduction in POMC mRNA does not occur in OVX rats when an LH surge is activated by estrogen/progesterone treatment (7). These studies were predicated on the notion that ß-endorphin derived from the POMC precursor negatively regulates GnRH cells and that reduction in expression of the precursor gene would reduce opioid tone at the time of the preovulatory GnRH/LH surge.

Melanocortins stimulate the reproductive axis in various species including rats, sheep, and humans (8). For example, αMSH induced ovulation in female rats pretreated with progesterone (9). Administration of a melanocortin antagonist reduced the magnitude of the LH surge in OVX steroid-primed rats (10), and treatment of

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.

doi: 10.1210/en.2009-0604 Received May 26, 2009. Accepted September 4, 2009.
First Published Online October 9, 2009

Abbreviations: aCSF, Artificial cerebrospinal fluid; ARC, arcuate nucleus; CV, coefficient of variation; DMH, dorsomedial hypothalamus; FG, FluoroGold; Kiss1, kisspeptin; LHA, lateral hypothalamic area; LV, lateral ventricular; MCR, melanocortin receptor; MTII, melanotan II; ORX, orexin; OVX, ovariectomized; PFA, perifornical area; POA, preoptic area; POMC, proopiomelanocortin; ZI, zona incerta.

human males with αMSH increased plasma LH levels (11). Moreover, γMSH stimulates GnRH release in immortalized GnRH cell lines and hypothalamic explants *in vitro* and plasma LH levels *in vivo* (12). Recent studies of lean OVX ewes showed that a melanocortin agonist can overcome the hypogonadotropic state of these animals, stimulating pulsatile LH secretion (13). Because the POMC cells are important in the regulation of energy balance (14, 15), these cells (and the melanocortins) could provide a mechanism by which energy status and reproduction are linked. Whether these POMC cells synapse directly with GnRH neurons is unknown; however, we have shown previously that very few cells from the ARC actually synapse with GnRH neurons in the ventral preoptic area (POA) (16). Conversely, others, using retrograde (17) and anterograde (18) tracers, detected input from the ARC to GnRH cells. These latter studies, however, report large injection volumes and do not limit the injection sites to the region of interest in which GnRH or ARC cells are located, and we believe an interneuronal pathway between the ARC cells and GnRH neurons is likely. Orexin (ORX) (19) and kisspeptin (20–23) have both been reported as positive regulators of the neuroendocrine reproductive axis, and both these cell types have demonstrated direct input to GnRH cells in the sheep (24, 25) and rodent (26). It is therefore possible that these cells act as a conduit for melanocortin feedback, but this is yet to be elucidated.

To further investigate the role of melanocortins in the regulation of reproduction, we carried out studies in the ewe, using a melanocortin agonist melanotan II (MTII). First, we performed lateral ventricular (LV) infusions of the agonist in ewes during the luteal phase of the estrous cycle, which is a period when gonadotropin secretion is held under negative feedback by the combined effects of estrogen and progesterone (27). Because we observed a stimulatory effect of the agonist on plasma LH levels, we then treated animals in the luteal phase of the cycle with the agonist and examined gene expression for prepro-ORX and kisspeptin (Kiss1) because these may act as neurotransmitter intermediaries to regulate the GnRH cells (24, 25). To determine whether melanocortin action can overcome photoperiodic suppression of the reproductive axis, we infused MTII (LV) into seasonally anestrous ewes to examine acute response in terms of LH secretion and possible stimulation of ovulation.

Materials and Methods

Animals

The experiments used adult Corriedale ewes, which were maintained on pasture or in feedlots under natural conditions. For intensive experimentation, the animals were housed in individual pens with natural lighting and had access to lucerne chaff and water *ad libitum*. The animals were conditioned to pen housing and handling for 1 wk before experimentation and LV cannulation was carried out at least 2 wk before experimentation as previously described (28). 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.

Experiment 1: effect of MTII treatment during the luteal phase of the estrous cycle on LH secretion

Eight adult Corriedale ewes received LV cannulae, as previously described (28), at least 1 month before use. Their estrous cycles were synchronized by two sequential im injections of 125 μg of synthetic luteolysin Cloprostenol (Estrumate; Pitman-Moore, Sydney, Australia) 10 d apart, and the experiment was performed 10 d after the second injection (late luteal phase of the estrous cycle). On the day before experimentation, the animals received an external jugular venous cannula (Tuta Healthcare, Sydney, New South Wales, Australia), which was kept patent with heparinized (50 U/ml) saline. The cannula was extended to the side of the animal pens with polyethylene tubing and closed with a three-way stopcock. Verification of the stage of cycle was by measurement of plasma progesterone levels (29), which were 4.1 ± 0.7 ng/ml. Serial blood sampling commenced at 0900 h and samples (5 ml) were taken every 10 min for 3 h before treatment and for 3 h during infusion. MTII (10 μg/h; n = 4) or artificial cerebrospinal fluid (aCSF) as vehicle (n = 4) was administered by LV infusion (55 μl/h) using MS16A Grasby minipumps (Graseby Medical Ltd., Gold Coast, Australia). Plasma was harvested and stored at −20 C until assayed for LH.

Experiment 2: retrograde tracing from the POA to the POMC and kisspeptin cells in the ARC and the kisspeptin cells of the dorsolateral POA

Retrograde labeling from the ventromedial POA was performed to determine whether ARC POMC and/or kisspeptin cells and POA kisspeptin cells project to the ventromedial POA, in which the majority of GnRH cells are found (30). Analysis of kisspeptin cells was included because of the possibility that MTII acts via these cells to affect GnRH secretion (*vide infra*). Adult Corriedale ewes (n = 4) received 75 nl injections of 4% Fluoro-Gold (FG; Fluorochrome Inc., Englewood, CO.) into the ventromedial POA as previously described (24). Animals were then returned to pasture for 3 wk to allow optimum retrograde transport of FG to the ARC in the brain. After this time, the animals were euthanized by iv injection of 20 ml sodium pentobarbital (Lethabarb; May and Baker Pty. Ltd., Melbourne, Victoria, Australia), and the brains were perfused and processed for immunohistochemistry as described previously (31). Frozen sections were cut in the coronal plane (40 μm), collected into cryoprotectant at −20 C until processed. For each animal, three sections representing rostral, middle, and caudal ARC and three sections throughout the POA were processed for fluorescent immunohistochemistry (*vide infra*).

Experiment 3: ORX and Kiss1 gene expression after MTII treatment

MTII stimulated LH secretion in experiment 1, but the results of experiment 2 showed that very few POMC or kisspeptin cells projected to the ventral POA. We repeated the treatments of experiment 1 in the same animals to ascertain whether MTII affected expression of genes for neuropeptides that may act as interneurons between POMC cells and GnRH cells, namely kisspeptin cells and ORX cells. After experiment 1, the ewes were allowed to progress through another estrous cycle and were in the luteal phase of the estrous cycle when they were randomly assigned to receive LV infusions of either MTII (10 μg/h) or vehicle (aCSF) for 20 h. This time frame was chosen to allow adequate time for possible changes in gene expression to occur. The brains of the animals were perfused with paraformaldehyde as previously described (32). The hypothalamus was dissected as a block and postfixed at 4 C in fixative containing 30% sucrose for 7 d. Frozen sections were cut in the coronal plane (20 μm), collected into cryoprotectant with 2% paraformaldehyde, and stored at −20 C until processed for *in situ* hybridization analysis of ORX and Kiss1 mRNA.

Experiment 4: MTII treatment to acyclic anestrous ewes

MTII treatment stimulated LH secretion in the breeding season, so we determined whether the melanocortin agonist could stimulate gonadotropin secretion and induce ovulation in seasonally acyclic anestrous ewes. Adult Corriedale ewes with LV cannulae were treated during November (anestrous season for this breed) and received LV infusions of either MTII (10 μg/h; n = 6) or vehicle (aCSF; n = 6). Before the experiment all ewes were treated with progesterone to prime the neuroendocrine system to effects of estrogen (33). This was carried out by the insertion of an intravaginal progesterone controlled delivery device (CIDR; InterAg, Hamilton, New Zealand). The CIDRs were removed and 24 h later the animals received either MTII or vehicle (aCSF) for 30 h (55 μl/h). Jugular blood samples were collected every 2 h from 4 h before infusion until 6 h afterward to monitor acute response in terms of LH levels. Then, from 28 to 35 h after the commencement of MTII infusion, additional blood samples were collected hourly to detect the occurrence of an LH surge. Finally, blood samples were taken every second day for 14 d for assay of plasma progesterone to determine whether ovulation had occurred with an ensuing corpus luteum.

Immunohistochemistry and FG mapping

The FG injection sites were localized using fluorescence microscopy (for FG) on sections counterstained with cresyl fast violet as previously described (31). Double-label immunohistochemistry was then performed on injection sites to ensure the spread of the injection had encompassed GnRH cell bodies. GnRH cells were identified using a monoclonal antibody against GnRH (dilution 1:2000; HU11B, courtesy of Professor H. Urbanski, Oregon Regional Primate Research, Beaverton, OR), and a goat antimouse Alexa 488 (Molecular Probes Inc., Eugene, OR) (dilution 1:500). FG injection sites were visualized using UV light filter (excitation 330–380 nm, barrier 420 nm). Animals with correct placement of FG injections within the POA were selected for further study. Anatomically matching sections representing rostral, middle, and caudal ARC and three anatomical matching sections throughout the POA from each animal were mounted onto superfrost slides and dried overnight. POMC-containing cells were identified using a γMSH primary antibody (Antibodies Australia, Melbourne, Australia) with specificity as previously described (34). Preabsorption with 0.5 mg/ml of the original peptide abolished all staining in the ovine ARC (data not shown). To detect kisspeptin, we used a polyclonal rabbit antibody against mouse kisspeptin-10 (dilution1:100,000; gift from A. Caraty, Institut National de la Recherche Agronomique, Nouzilly, France) (35) previously validated for use in sheep tissues (34). Secondary antibodies for γMSH and kisspeptin staining were anti-guinea pig and anti-rabbit respectively, conjugated to Alexa 546 (1:500, Molecular Probes). After staining for γMSH or kisspeptin, the sections were then labeled for FG (rabbit polyclonal antibody; dilution 1:2000; Chemicon International, Sydney, Australia) using a goat anti-rabbit conjugated to Alexa 488 (1:500) as previously reported (31). Sections were coverslipped using antifade medium (Dako, Botany Bay, New South Wales, Australia). A microscope (BMX50; Olympus, Tokyo, Japan) equipped with a UV light filter (excitation 330–380nm, barrier 420 nm) was used to map the distribution of FG cells, kisspeptin cells, and melanocortin cells, including the counting of cells colocalizing FG and peptide, using the MD plot system (version 5.0; AccuStage, Shoreview, MN.).

In situ hybridization

In situ hybridization (experiment 3) was performed using ^{35}S-deoxyuridine 5-triphosphate-labeled riboprobes according to a described protocol (36, 37). The cDNA and plasmid inserts used were a 375-base ovine Kiss1 gene and a 207-base ovine prepro-ORX gene, which were both inserted into a pGem T-easy plasmid. The amplification and linearization of plasmid DNA was performed using standard techniques (38). When analyzing ORX gene expression, two anatomically matching sections across the dorsomedial hypothalamus (DMH), perifornical (PFA), zona incerta (ZI), and lateral hypothalamic (LHA) areas were chosen for each animal because this is where the majority of ORX-expressing cells are located in the ewe (24). For analysis of Kiss1 expression, three sections from each ewe were taken to represent rostral, middle, and caudal regions of the ARC, and three to five sections through the dorsolateral POA were chosen for analysis of Kiss1 expression in this region. After hybridization, the slides were dipped in K5 photographic emulsion (Ilford Imaging, Melbourne, Australia) and kept at 4 C in the dark and developed after 4 d (ORX), 7 d (Kiss1 ARC), or 9 d (Kiss1 POA). Sections were then counterstained with 1% cresyl violet, dehydrated, and coverslipped using DePex plastic resin (Grale Scientific, Melbourne, 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 when there was a clearly discernible nucleus. Computer-assisted grain counting was performed under bright light field at ×40 using an HC-2000 high-resolution digital camera (Fuji, Tokyo, Japan) and Analytical Imaging system 3.0 software (Image Pro Plus, Media Cybernetics, Silver Spring, MD), as previously described (32). The number of silver grains per cell for Kiss1 mRNA was estimated in 40 cells/section in the ARC and 20 cells/section in the POA. ORX expression was analyzed in the DMH, PFA, ZI, and LHA, and silver grains per cell was estimated in 40 cells/region.

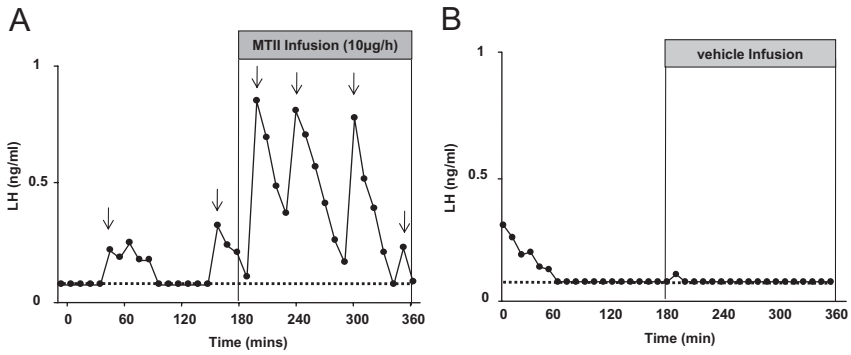

FIG. 1. Representative profile of the effect of LV infusion of MTII (10 μg/h; n = 4) (A) or aCSF (n = 4) (B) for 3 h on plasma LH levels from ewes in the luteal phase of the estrous cycle. Plasma samples were collected every 10 min for 3 h before and after the start of infusion. *Dotted baseline* is representative of LH assay sensitivity (0.09 ng/ml). *Arrows* indicated significant pulses.

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 sensitivity of the LH assays was 0.1 ng/ml (experiment 1) and 0.2 ng/ml (experiment 4). For all oLH assays, the interassay coefficient of variation (CV) was less than 15% and the intraassay CV less than 8%. An LH pulse was defined as having occurred when the assay value of a given sample exceeded the assay value of the previous sample by at least 3 SD as well as other criteria detailed previously (40).

Plasma progesterone levels were measured in a single assay based on the method of (41), after hexane extraction of 100 μl plasma with a sensitivity of 0.1 ng/ml and an intraassay CV of 5%.

Statistics

Data are presented as means (±SEM). Hormone data were analyzed by repeated-measures ANOVA, with least significant differences as a *post hoc* test. Measures of mRNA levels were analyzed by one-way independent-measures ANOVA.

Results

Experiment 1: MTII treatment during the luteal phase of the estrous cycle

Compared with preinfusion values, MTII treatment increased pulsatile LH secretion (Fig, 1A) by increasing mean plasma LH concentration ($P < 0.05$) and LH pulse frequency ($P < 0.05$), with no effect on LH pulse amplitude (Table 1). Infusion of aCSF had no effect on plasma LH levels (Fig. 1B). All values obtained below the sensitivity of the LH RIA were taken at the sensitivity threshold of 0.1 ng/ml.

Experiment 2: projections of kisspeptin and melanocortin cells to the ventromedial POA (retrograde labeling)

All FG injections sites were located in the ventromedial POA and positioned around the third ventricle (Fig. 2). Retrograde labeling of POMC and kisspeptin cells in the ARC was rarely seen, so that 1 ± 0.6% of POMC (γMSH immunoreactive) and 1.9 ± 0.2% of kisspeptin cells were observed to be colabeled with FG. In the dorsolateral POA, 76.4 ± 5.1% (average of four animals) kisspeptin cells were retrogradely labeled with FG.

Experiment 3: ORX and Kiss1 gene expression after MTII treatment

MTII treatment increased ORX mRNA expression in the DMH (Fig. 3) by increasing the number of detectable cells ($P < 0.05$) and the level of expression (grains per cell, $P < 0.01$), but there was no effect of MTII treatment on ORX gene expression in the PFA, ZI or LHA (Fig 3, A and B).

MTII treatment reduced ($P < 0.05$) Kiss1 mRNA expression in the ARC and increased ($P < 0.05$) Kiss1 mRNA expression in the POA. In both cases this was due to a change in the level of expression per cell and not the number of cells detected (Fig. 4).

Experiment 4: MTII treatment of acyclic anestrous ewes

Although plasma LH levels rose immediately after the start of infusion of MTII, from nondetectable to 1.0 ± 0.4 ng/ml, this rise was not statistically significant ($P < 0.09$).

TABLE 1. Plasma LH levels prior to and after infusion of MTII or vehicle in ewes during the luteral phase of the estrous cycle

	Mean LH (ng/ml)		LH pulse frequency (pulses/h)		LH pulse amplitude (ng/ml)	
	Before	After	Before	After	Before	After
MTII	0.16 ± 0.16	0.46 ± 0.09[a]	0.42 ± 0.08	1.08 ± 0.16[a]	0.73 ± 0.22	0.68 ± 0.03
Vehicle	0.10 ± 0.08	0.05 ± 0.04	0.250.25 ± 0.25	0.17 ± 0.09	0.21 ± 0.21	0.25 ± 0.18

Analysis of mean LH concentration (nanograms per milliliter), LH pulse frequency (pulses per hour), and LH pulse amplitude (nanograms per milliliter) before and after MTII or aCSF intracerebroventricular infusion to ewes (n = 4/group) in the luteal phase of the estrous cycle. Data are means ± SEM.

[a] $P < 0.05$ compared with preinfusion values.

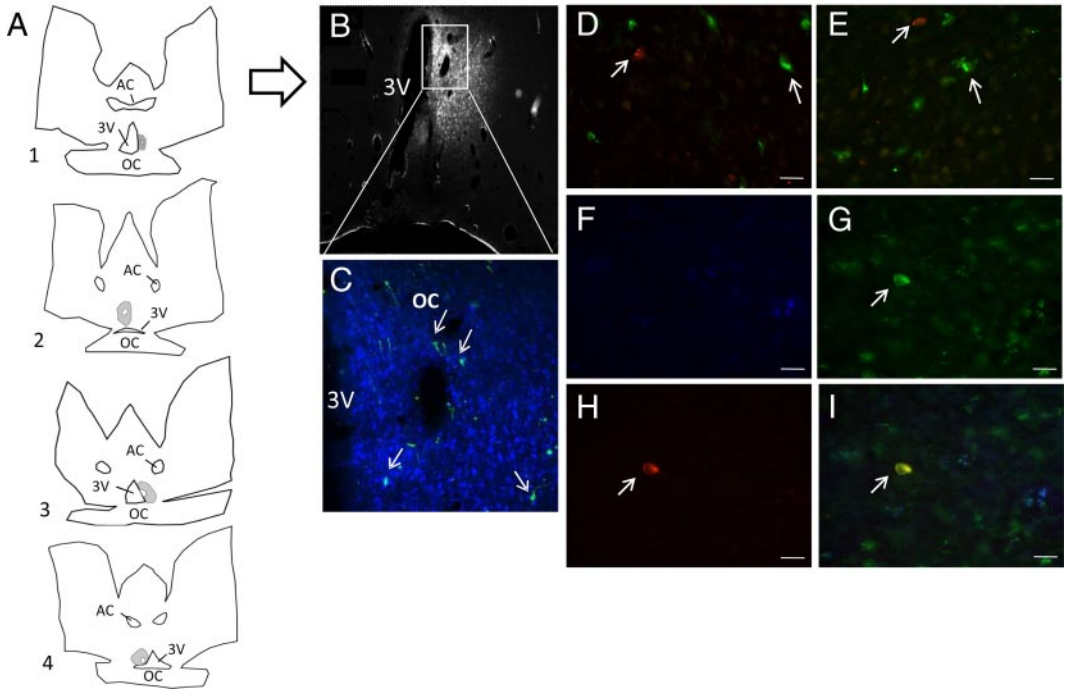

FIG. 2. FG injections into the ventral POA in which GnRH neurons are located. Schematic representation of injection sites from all four animals (A). *Inner white circle* indicates injection site, whereas the *gray surrounding shading* specifies the extent of the spread of tracer. Photomicrograph example of FG injection site in the POA (B), overlapping GnRH neurons (indicated by *arrows*; C). Retrogradely labeled FG cells (*red*) in the ARC do not colocalize with kisspeptin (*green*; D) or POMC cells (*green*; E). More than 70% of kisspeptin cells (*green*) in the dorsolateral POA colocalize with FG cells (*red*; F–I). *Scale bar*, 20 μm. OC, Optic chiasm; AC, anterior commissure; 3V ,third ventricle. In D–H, *arrows* to *red* objects indicate FG-labeled cells, and *arrows* to *green* objects indicate kisspeptin (D) and POMC (E). A kisspeptin cell in the dorsolateral POA (G; *arrow*) is labeled with FG (H) and dual-labeling (*yellow*) of this cell is shown in I (*arrow*).

Plasma LH levels were significantly ($P < 0.05$) elevated at the 4-h time point. No discernible LH surges were detected over the period during which we sampled (Fig. 5), nor was there any evidence of an increase in mean postinfusion progesterone levels between the two treatment groups (MTII treated: 0.1 ± 0.04 ng/ml; vehicle: 0.3 ± 0.10 ng/ml), which would have indicated active corpora lutea after ovulation. All values obtained below the sensitivity of the LH RIA were taken at the sensitivity threshold of 0.2 ng/ml.

Discussion

These studies substantiate a growing body of evidence to indicate that the melanocortin system within the brain acts to stimulate the reproductive axis. The present results support other recent data obtained in lean hypogonadotropic ewes to suggest that the means by which leptin activates the GnRH/gonadotropin axis is through the melanocortin system (13). During the luteal phase, gonadotropin levels are suppressed due to progesterone/estrogen negative feedback, but melanocortin treatment can overcome this negative clamp. The present study corroborates earlier data (16), which showed that there is relatively poor direct input to the ventromedial POA from the arcuate nucleus. We expanded this earlier work to show that the melanocortin and Kiss1 cells of the ARC do not provide substantial input to the ventromedial POA. There may, however, be an indirect pathway to GnRH cells from the cells of the ARC (16). Expression of ORX mRNA in cells of the DMH and expression of Kiss1 mRNA in cells of the dorsolateral POA was increased after melanocortin treatment to luteal phase ewes, so these cells may act as intermediary neurons to transmit melanocortin signals to the GnRH neurons.

We have shown that MTII treatment can overcome the sex steroid-negative feedback effect on GnRH secretion (43) during the luteal phase of the ewe. Earlier reports showed that POMC mRNA in the ARC of the ewe brain (2), and total αMSH protein levels in the mediobasal hypothalamus and POA of the rat brain (44) remains unchanged across the estrous cycle. These studies, however, did not take into account the marked differences in the biological properties of the different forms of αMSH. Acetylated αMSH is much more potent the des-acetyl form (45, 46), and levels of the former are reduced in conditions of low gonadotropin production in lean animals. Importantly, this recent work showed that the major change in melanocortin status that occurs with altered reproductive state is the level of αMSH in the terminal beds of melanocortin neurons and not in the ARC. To gain

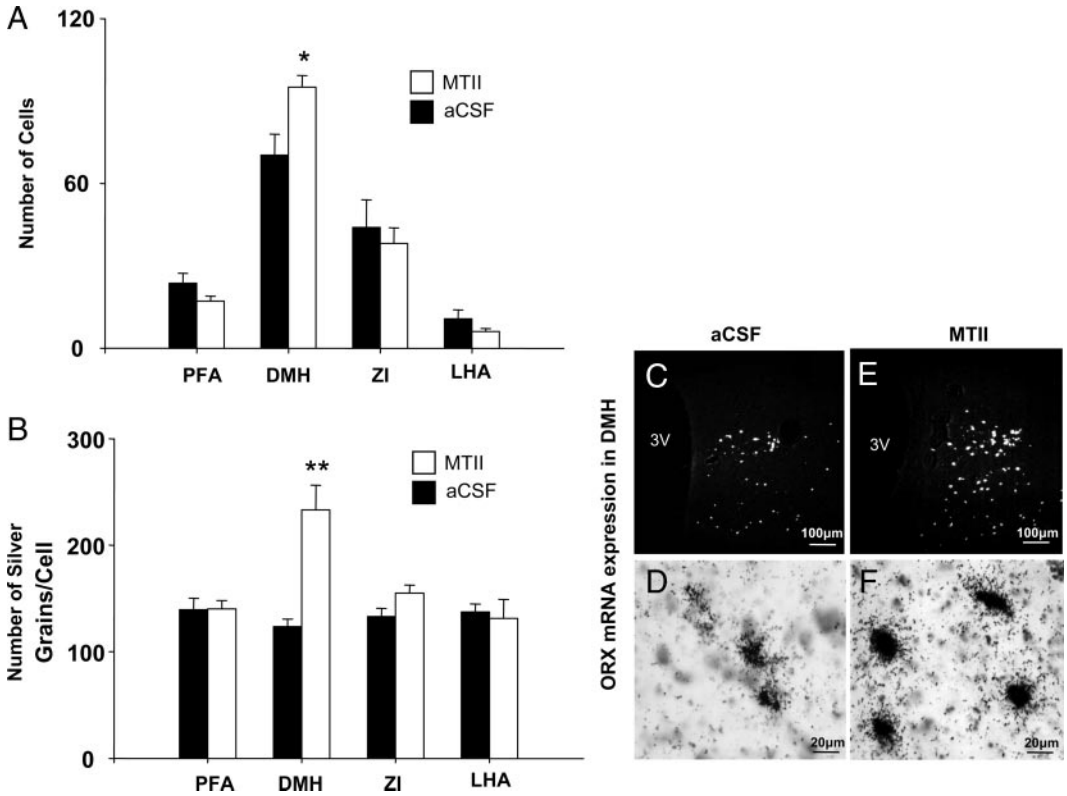

FIG. 3. Effect of LV infusion of aCSF (n = 4) or MTII (10 μg/h; n = 4) for 20 h on ORX gene expression in the PFA, DMH, ZI, and LHA, expressed by the average number of cells per section and the number of silver grains per cell (A and B). Dark-field (×10) and bright-field (×40) photomicrographs of ORX expression in the DMH (C–F). 3V, Third ventricle. *Solid bar*, aCSF treatment; *open bar*, MTII treatment. Data are means ± SEM. *, $P < 0.05$; **, $P < 0.01$ compared with aCSF treatment.

a more comprehensive understanding of the operation of the melanocortin system across the estrous cycle, further investigation of the type of melanocortin that prevails at each stage is required. Plasma levels of acetylated αMSH in humans are at highest concentration during the late follicular phase, when gonadotropin secretion is correspondingly high (47). The present result obtained with MTII treatment of ewes in the luteal phase of the estrous cycle indicates that melanocortin signaling may be suppressed at this time. This may be due to an effect on the production of acetylated αMSH, but there may also be effects on production of β- and/or γMSH, which also activate the melanocortin receptor (MCR)-3 and -4 (48, 49). Appropriate proteomic analysis is required to determine whether there is any change in the levels and forms of melanocortins in terminal beds in relevant regions of the hypothalamus and POA across the estrous cycle.

We measured ORX gene expression in animals treated with MTII because of earlier studies showing that approximately one third of GnRH cells in the ovine brain appear to receive direct input from ORX cells in the lateral hypothalamus (24). ORX mRNA was up-regulated in the DMH after melanocortin treatment of luteal phase ewes. Because this treatment also caused increased plasma LH levels, the data are consistent with earlier work in steroid primed OVX rats showing an increase in LH secretion after ORX treatment (19). Our earlier anatomical study, however, showed that ORX cells of the DMH do not project to the ventromedial POA in the sheep (24). To reconcile these findings and invoke a role for these ORX cells in the melanocortin stimulation of the reproductive axis, it is necessary to consider the possibility of serial connection to GnRH cells. It is possible that the ORX cells of the DMH project to some other rostrally located area to relay information to GnRH neurons. This pathway may involve interneurons in the bed nucleus of the stria terminalis and/or the median preoptic nucleus/lateral preoptic area (50). It is also possible that these ORX cells in the DMH act via the kisspeptin cells located in the dorsallateral POA (35). Support for the notion that the ORX cells of the DMH form at least part of the response to a melanocortin agonist, that is relayed to GnRH cells is the fact that this nucleus is highly innervated by αMSH-containing axons and axon terminals originating from the ARC in the rat (51), and ablation of the ARC results in a discernible reduction of αMSH fibers in the DMH (52). Finally, melanocortin receptors (MC3R and MC4R) are found in the DMH in the ewe (53). An alternative rationale for the increase in ORX gene expression with MTII treatment may be related to appetite regulation, although this

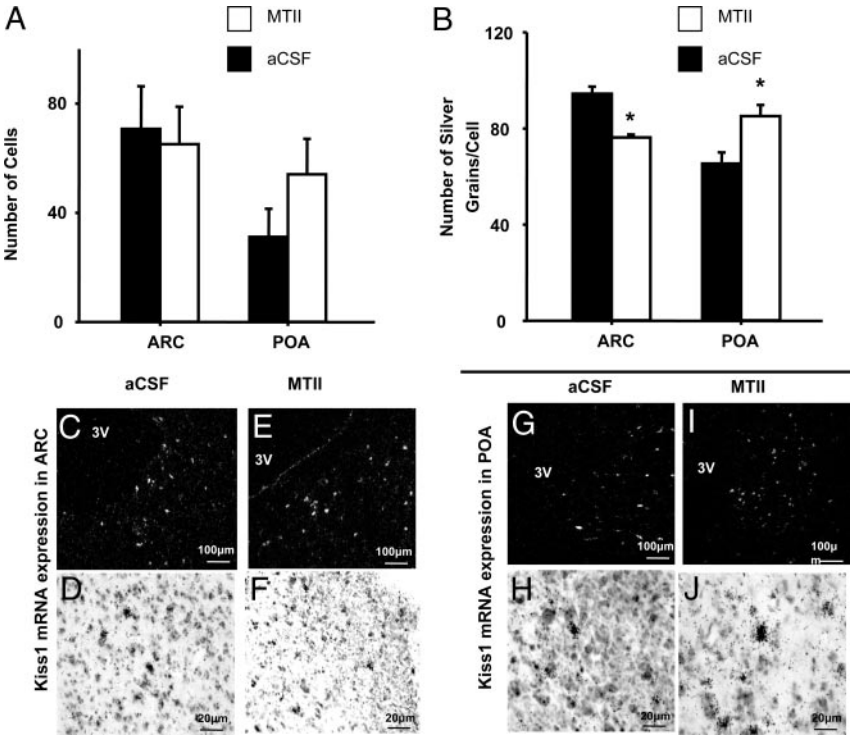

FIG. 4. Effect of LV infusion of aCSF (n = 4) or MTII (10 μg/h; n = 4) for 20 h on Kiss1 gene expression in the ARC and POA of the ewe, expressed by the average number of cells per section and the number of silver grains per cell (A and B). Dark-field (×10) and bright-field (×40) photomicrograph representations of ARC (C–F) and POA (F–J). *Solid bar*, aCSF treatment; *open bar*, MTII treatment. Data are means ±SEM *, $P < 0.05$ compared with aCSF treatment. 3V, Third ventricle.

would seem contradictory because ORX stimulates food intake (54) and melanocortins (55) have the reverse effect.

In sheep in the luteal phase of the cycle, MTII treatment reduced Kiss1 mRNA levels in the ARC but increased expression in the POA. Sex steroids, particularly estradiol-17β, negatively regulate Kiss1 mRNA expression in the ARC (56) and up-regulate Kiss1 mRNA expression in the POA (25), leading to the perceived role of kisspeptin in facilitating both negative and positive feedback responses of sex steroids to GnRH neurons (57). It is important, therefore, to take account of the likely rise in estradiol-17β levels, which result from the increase in LH secretion after melanocortin treatment. It is unknown whether the increase in Kiss1 mRNA in the POA and the decrease in the ARC is a direct effect of melanocortin treatment or is a consequence of increased estradiol-17β levels. It has been suggested that the MC4R is the principal melanocortin receptor involved in stimulating the reproductive axis (10), and this subtype is expressed at a high level in the POA but not the ARC of the ewe (53). Furthermore, virtually all of the Kiss1 cells of the ARC express estrogen receptor-α, but only 50% of the same cell type in the POA express the receptor (35). This leads us to tentatively conclude that melanocortins activate the Kiss1 cells of the POA directly, but the decrease in Kiss1 expression seen in the ARC may be due to a rise in sex steroid levels.

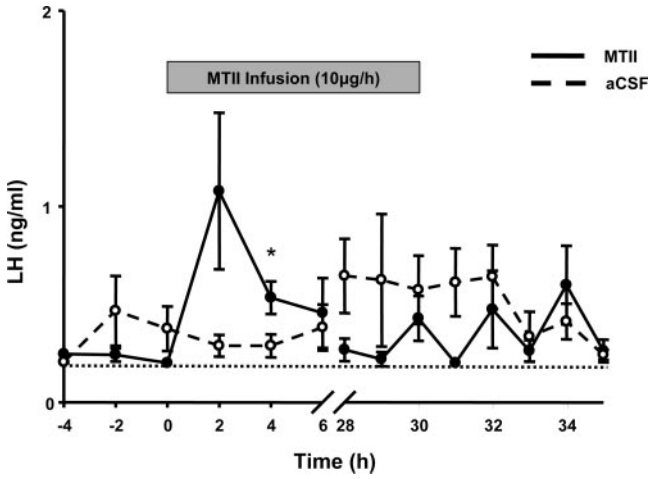

FIG. 5. Effect of LV infusion of aCSF (n = 6) or MTII (10 μg/h; n = 6) for 30 h on LH secretion in anestrous ewes. Blood samples were collected every 2 h. *Continuous line*, MTII-treated ewes; *dashed line*, aCSF-treated ewes; *dotted baseline*, LH assay sensitivity (0.2 ng/ml). Data are means ± SEM. *, $P < 0.05$ compared with aCSF LH concentration.

MTII treatment caused a small increase in LH levels in anestrous ewes, but this did not translate into an LH surge, ovulation, or the formation of corpora lutea. In this experiment, the major objective was to determine whether there was a positive feedback response to the treatment, causing ovulation. Plasma LH levels in samples collected before and shortly after commencement of treatment suggest that MTII treatment can at least partly overcome the enhanced negative feedback effect of estrogen that prevails during anestrus (58). A more intense series of blood samples around this treatment may provide more detailed data on this point, but it is clear from the present results that MTII treatment cannot reverse the acyclic condition of anestrus. The relatively short-lived response to MTII in these animals may have been due to stimulation of ovarian function, sufficient to increase estradiol-17β secretion and effect a negative feedback response on GnRH/LH secretion, thus preventing sustained activation of the reproductive axis and ovulation. Preovulatory-like LH surges did not occur in any of the treated ewes, nor was there any evidence of ovulation. It is possible that there is reduced responsiveness to melanocortins in the anestrous season. POMC mRNA is increased in anestrous ewes (59) and

rams (60), but it remains to be determined whether MC3R and MC4R expression is changes with season in the ewe.

The present study confirms previous observations with anterograde tracing, which indicated relatively poor direct input to the ventromedial POA from the ARC (16). We found that very few POMC or Kiss1 cells of the ARC were retrogradely labeled when FG was placed in the ventral POA. Our earlier results (16) were contradicted by those of another group, using relatively large injections of retrograde tracer placed in various regions of the POA and diagonal band of Broca (18), but our present results confirm our earlier findings. We also demonstrate, in corroboration with previous data from our laboratory, which showed GnRH input from lateral regions of the POA (50), that more than 70% of dorsolateral POA kisspeptin cells were retrogradely labeled with FG injected into the ventromedial POA. This suggests that the observed input to GnRH cells from kisspeptin cells in the ovine brain arises almost exclusively from the population of kisspeptin cells in the dorsolateral POA and not from the population located in the ARC. Thus, stimulation of the GnRH cells from any elements in the ARC, especially kisspeptin and/or melanocortins, is likely to be indirect, involving at least one interneuron. Although only few projections were noted from the ARC Kiss1 and POMC cells to the ventromedial POA in which GnRH cells are found, it does not preclude the projection of these cells to the dorsolateral POA to activate Kiss1 neurons.

In support of this notion, Kiss1 mRNA was up-regulated in the POA after melanocortin treatment in the luteal phase. The functional significance of the Kiss1 cells in the POA still remains to be fully elucidated in this species, but it has been proposed that these may act in a similar manner to the Kiss1 cells in the anteroventral periventricular nucleus of the rodent, which positively regulate GnRH/LH secretion during the preovulatory LH surge (57). In this regard, it is notable that Kiss1 mRNA in the POA is up-regulated in the periovulatory phase in monkeys and the late follicular phase in sheep (Smith, J. T., and I. J, Clarke, unpublished observations). It is therefore plausible that the Kiss1 cells of the POA are involved in the positive regulation of GnRH neurons and act as an interneuronal pathway for the Kiss1 and/or melanocortin cells of the ARC. Alternatively, GnRH secretion may be regulated by melanocortins and/or kisspeptin cells at the level of the median eminence. Recent studies suggest that there is some association between kisspeptin fibers and GnRH fibers in the median eminence (61), and several reports demonstrate that POMC cells project to the median eminence (62). On the other hand, there is no substantive evidence for synaptic input to GnRH axons and terminals within the median eminence, and communication may be by volume transmission (42, 64, 65).

In conclusion, this study adds further impetus to the notion that the melanocortins are of fundamental importance to the normal reproductive process. We have reinforced the fact that melanocortin signaling positively regulates GnRH cells because MTII has a convincing effect to increase pulsatile LH secretion in luteal phase ewes and is likely to increase basal LH levels during seasonal anestrous. Evidence is presented that this effect may be transmitted via ORX and/or kisspeptin cells in the DMH and POA, respectively. We further show that direct kisspeptin stimulation of GnRH cells occurs from the population of kisspeptin cells located in the dorsolateral POA and that kisspeptin cells of the ARC likely regulate GnRH cells indirectly. Understanding the involvement of the melanocortin system in stimulating the reproductive axis builds evidence to support the use of melanocortins as a therapeutic target in times of reproductive suppression.

Acknowledgments

We thank Mr. B. Doughton, Ms. L. Morrish, and Ms. E. Gleeson for their technical assistance; and Dr. A. F. Parlow (National Hormone and Peptide Program, National Institute of Diabetes and Digestive and Kidney Diseases, Torrance, CA) for assay reagents.

Address all correspondence and requests for reprints to: Prof. Iain Clarke, PhD, Department of Physiology, P.O. Box 13F, Monash University, Clayton, Victoria 3800, Australia. E-mail: iain.clarke@med.monash.edu.au.

This work was supported by a grant from the National Health and Medical Research Council of Australia (to I.J.C.).

References

1. **Pritchard LE, Turnbull AV, White A** 2002 Pro-opiomelanocortin processing in the hypothalamus: impact on melanocortin signalling and obesity. J Endocrinol 172:411–421
2. **Walsh JP, Rao A, Simmons DM, Clarke IJ** 1998 Proopiomelanocortin mRNA levels in ovine hypothalamus are not reduced at the time of the preovulatory luteinising hormone surge. J Neuroendocrinol 10:803–808
3. **Wise PM, Scarbrough K, Weiland NG, Larson GH** 1990 Diurnal pattern of proopiomelanocortin gene expression in the arcuate nucleus of proestrous, ovariectomized, and steroid-treated rats: a possible role in cyclic luteinizing hormone secretion. Mol Endocrinol 4:886–892
4. **Bohler Jr HC, Tracer H, Merriam GR, Petersen SL** 1991 Changes in proopiomelanocortin messenger ribonucleic acid levels in the rostral periarcuate region of the female rat during the estrous cycle. Endocrinology 128:1265–1269
5. **Tong Y, Zhao HF, Labrie F, Pelletier G** 1990 Regulation of proopiomelanocortin messenger ribonucleic acid content by sex steroids in the arcuate nucleus of the female rat brain. Neurosci Lett 112:104–108

6. **Wilcox JN, Roberts JL** 1985 Estrogen decreases rat hypothalamic proopiomelanocortin messenger ribonucleic acid levels. Endocrinology 117:2392–2396
7. **Petersen SL, Keller ML, Carder SA, McCrone S** 1993 Differential effects of estrogen and progesterone on levels of POMC mRNA levels in the arcuate nucleus: relationship to the timing of LH surge release. J Neuroendocrinol 5:643–648
8. **Schiöth HB, Watanobe H** 2002 Melanocortins and reproduction. Brain Res Brain Res Rev 38:340–350
9. **Alde S, Celis ME** 1980 Influence of α-melanotropin on LH release in the rat. Neuroendocrinology 31:116–120
10. **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
11. **Limone P, Calvelli P, Altare F, Ajmone-Catt P, Lima T, Molinatti GM** 1997 Evidence for an interaction between α-MSH and opioids in the regulation of gonadotropin secretion in man. J Endocrinol Invest 20:207–210
12. **Stanley SA, Davies S, Small CJ, Gardiner JV, Ghatei MA, Smith DM, Bloom SR** 2003 γ-MSH increases intracellular cAMP accumulation and GnRH release *in vitro* and LH release *in vivo*. FEBS Lett 543: 66–70
13. **Backholer K, Bowden M, Gamber K, Bjorbaek C, Iqbal J, Clarke IJ** Propiomelanocortin, melanocortin, and reproductive function in lean body condition. Program of the 90th Annual Meeting of The Endocrine Society, San Francisco, CA, 2008 (Abstract P1-690)
14. **Mizuno TM, Kleopoulos SP, Bergen HT, Roberts JL, Priest CA, Mobbs CV** 1998 Hypothalamic pro-opiomelanocortin mRNA is reduced by fasting and [corrected] in ob/ob and db/db mice, but is stimulated by leptin. Diabetes 47:294–297
15. **McShane TM, Petersen SL, McCrone S, Keisler DH** 1993 Influence of food restriction on neuropeptide-Y, proopiomelanocortin, and luteinizing hormone-releasing hormone gene expression in sheep hypothalami. Biol Reprod 49:831–839
16. **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
17. **Goubillon M, Delaleu B, Tillet Y, Caraty A, Herbison AE** 1999 Localization of estrogen-receptive neurons projecting to the GnRH neuron-containing rostral preoptic area of the ewe. Neuroendocrinology 70:228–236
18. **Goubillon ML, Caraty A, Herbison AE** 2002 Evidence in favour of a direct input from the ventromedial nucleus to gonadotropin-releasing hormone neurones in the ewe: an anterograde tracing study. J Neuroendocrinol 14:95–100
19. **Pu S, Jain MR, Kalra PS, Kalra SP** 1998 Orexins, a novel family of hypothalamic neuropeptides, modulate pituitary luteinizing hormone secretion in an ovarian steroid-dependent manner. Regul Pept 78:133–136
20. **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
21. **Smith JT, Clifton DK, Steiner RA** 2006 Regulation of the neuroendocrine reproductive axis by kisspeptin-GPR54 signaling. Reproduction 131:623–630
22. **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
23. **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
24. **Iqbal J, Pompolo S, Sakurai T, Clarke IJ** 2001 Evidence that orexin-containing neurones provide direct input to gonadotropin-releasing hormone neurones in the ovine hypothalamus. J Neuroendocrinol 13:1033–1041
25. **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 connections to gonadotropin-releasing hormone neurons in the brain: a novel medium for seasonal breeding in the sheep. Endocrinology 149:5770–5782
26. **Campbell RE, Grove KL, Smith MS** 2003 Gonadotropin-releasing hormone neurons coexpress orexin 1 receptor immunoreactivity and receive direct contacts by orexin fibers. Endocrinology 144: 1542–1548
27. **Karsch FJ, Cummins JT, Thomas GB, Clarke IJ** 1987 Steroid feedback inhibition of pulsatile secretion of gonadotropin-releasing hormone in the ewe. Biol Reprod 36:1207–1218
28. **Henry BA, Dunshea FR, Gould M, Clarke IJ** 2008 Profiling postprandial thermogenesis in muscle and fat of sheep and the central effect of leptin administration. Endocrinology 149:2019–2026
29. **Scott CJ, Pereira AM, Tilbrook AJ, Rawson JA, Clarke IJ** 2001 Changes in preoptic and hypothalamic levels of progesterone receptor mRNA across the oestrous cycle of the ewe. J Neuroendocrinol 13:401–406
30. **Lehman MN, Robinson JE, Karsch FJ, Silverman AJ** 1986 Immunocytochemical localization of luteinizing hormone-releasing hormone (LHRH) pathways in the sheep brain during anestrus and the mid-luteal phase of the estrous cycle. J Comp Neurol 244:19–35
31. **Qi Y, Iqbal J, Oldfield BJ, Clarke IJ** 2008 Neural connectivity in the mediobasal hypothalamus of the sheep brain. Neuroendocrinology 87:91–112
32. **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
33. **Caraty A, Skinner DC** 1999 Progesterone priming is essential for the full expression of the positive feedback effect of estradiol in inducing the preovulatory gonadotropin-releasing hormone surge in the ewe. Endocrinology 140:165–170
34. **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
35. **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
36. **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
37. **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
38. **Sambrook J, Fritsch E, Maniatis T** 1989 Molecular cloning, a laboratory manual. 2nd ed. Cold Spring Harbor, NY: Cold Sping Harbor Laboratory Press
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. **Clarke IJ, Cummins JT** 1985 Increased gonadotropin-releasing hormone pulse frequency associated with estrogen-induced luteinizing hormone surges in ovariectomized ewes. Endocrinology 116:2376–2383

41. **Deayton JM, Young IR, Thorburn GD** 1993 Early hypophysectomy of sheep fetuses: effects on growth, placental steroidogenesis and prostaglandin production. J Reprod Fertil 97:513–520
42. **Yin W, Mendenhall JM, Bratton SB, Oung T, Janssen WG, Morrison JH, Gore AC** 2007 Novel localization of NMDA receptors within neuroendocrine gonadotropin-releasing hormone terminals. Exp Biol Med (Maywood) 232:662–673
43. **Clarke IJ, Thomas GB, Yao B, Cummins JT** 1987 GnRH secretion throughout the ovine estrous cycle. Neuroendocrinology 46:82–88
44. **Khorram O, Bedran de Castro JC, McCann SM** 1985 The effect of the estrous cycle and estrogen on the release of immunoreactive α-melanocyte-stimulating hormone. Peptides 6:503–508
45. **Abbott CR, Rossi M, Kim M, AlAhmed SH, Taylor GM, Ghatei MA, Smith DM, Bloom SR** 2000 Investigation of the melanocyte stimulating hormones on food intake. Lack of evidence to support a role for the melanocortin-3-receptor. Brain Res 869:203–210
46. **Tsujii S, Bray GA** 1989 Acetylation alters the feeding response to MSH and β-endorphin. Brain Res Bull 23:165–169
47. **Mauri A, Martellotta MC, Melis MR, Caminiti F, Serri F, Fratta W** 1990 Plasma α-melanocyte-stimulating hormone during the menstrual cycle in women. Horm Res 34:66–70
48. **Harrold JA, Widdowson PS, Williams G** 2003 β-MSH: a functional ligand that regulated energy homeostasis via hypothalamic MC4-R? Peptides 24:397–405
49. **Gantz I, Konda Y, Tashiro T, Shimoto Y, Miwa H, Munzert G, Watson SJ, DelValle J, Yamada T** 1993 Molecular cloning of a novel melanocortin receptor. J Biol Chem 268:8246–8250
50. **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
51. **Jacobowitz DM, O'Donohue TL** 1978 α-Melanocyte stimulating hormone: immunohistochemical identification and mapping in neurons of rat brain. Proc Natl Acad Sci USA 75:6300–6304
52. **Homma A, Li HP, Hayashi K, Kawano Y, Kawano H** 2006 Differential response of arcuate proopiomelanocortin- and neuropeptide Y-containing neurons to the lesion produced by gold thioglucose administration. J Comp Neurol 499:120–131
53. **Iqbal J, Pompolo S, Dumont LM, Wu CS, Mountjoy KG, Henry BA, Clarke IJ** 2001 Long-term alterations in body weight do not affect the expression of melanocortin receptor-3 and -4 mRNA in the ovine hypothalamus. Neuroscience 105:931–940
54. **Sartin JL, Dyer C, Matteri R, Buxton D, Buonomo F, Shores M, Baker J, Osborne JA, Braden T, Steele B** 2001 Effect of intracerebroventricular orexin-B on food intake in sheep. J Anim Sci 79:1573–1577
55. **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
56. **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
57. **Smith JT** 2009 Sex steroid control of hypothalamic Kiss1 expression in sheep and rodents: comparative aspects. Peptides 30:94–102
58. **Legan SJ, Karsch FJ, Foster DL** 1977 The endocrin control of seasonal reproductive function in the ewe: a marked change in response to the negative feedback action of estradiol on luteinizing hormone secretion. Endocrinology 101:818–824
59. **Clarke IJ, Scott CJ, Rao A, Pompolo S, Barker-Gibb ML** 2000 Seasonal changes in the expression of neuropeptide Y and pro-opiomelanocortin mRNA in the arcuate nucleus of the ovariectomized ewe: relationship to the seasonal appetite and breeding cycles. J Neuroendocrinol 12:1105–1111
60. **Anukulkitch C, Rao A, Dunshea FR, Blache D, Lincoln GA, Clarke IJ** 2007 Influence of photoperiod and gonadal status on food intake, adiposity, and gene expression of hypothalamic appetite regulators in a seasonal mammal. Am J Physiol Regul Integr Comp Physiol 292:R242–R252
61. **Ramaswamy S, Guerriero KA, Gibbs RB, Plant TM** 2008 Structural interactions between kisspeptin and GnRH neurons in the mediobasal hypothalamus of the male rhesus monkey (*Macaca mulatta*) as revealed by double immunofluorescence and confocal microscopy. Endocrinology 149:4387–4395
62. **Conover CD, Kuljis RO, Rabii J, Advis JP** 1993 β-Endorphin regulation of luteinizing hormone-releasing hormone release at the median eminence in ewes: immunocytochemical and physiological evidence. Neuroendocrinology 57:1182–1195
63. **Kawakami S, Ichikawa M, Murahashi K, Hirunagi K, Tsukamura H, Maeda K** 1998 Excitatory amino acids act on the median eminence nerve terminals to induce gonadotropin-releasing hormone release in female rats. Gen Comp Endocrinol 112:372–382
64. **Durrant AR, Plant TM** 1999 A study of the gonadotropin releasing hormone neuronal network in the median eminence of the rhesus monkey (*Macaca mulatta*) using a post-embedding immunolabelling procedure. J Neuroendocrinol 11:813–821