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Photoperiod effects on gene expression for hypothalamic appetite-regulating peptides and food intake in the ram

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Clarke, Iain J., Alexandra Rao, Yves Chilliard, Carole Delavaud, and Gerald A. Lincoln. Photoperiod effects on gene expression for hypothalamic appetite-regulating peptides and food intake in the ram. *Am J Physiol Regul Integr Comp Physiol* 284: R101–R115, 2003. First published September 27, 2002; 10.1152/ajpregu.00424.2002.—Relationship between voluntary food intake (VFI) and gene expression for appetite-regulating peptides was examined in the brains of Soay rams under contrasting photoperiods. Two groups ($n = 8$) were subjected to alternating block long-day (LD) and short-day photoperiods (SD) over a period of 42 wk to entrain long-term cycles in VFI. Five animals from each group were killed 18 wk into LD or SD, and the brains were collected for in situ hybridization studies. VFI was fourfold higher under LD compared with SD. Body weight, abdominal fat, or plasma leptin levels were similar under LD and SD. LD animals were in positive energy balance and sexually inactive, and SD animals were in negative energy balance and sexually active. Neuropeptide Y (NPY) mRNA levels were higher in the arcuate nucleus (ARC) under LD, and pro-opiomelanocortin expression was lower under LD. Leptin receptor (Ob-Rb) was higher in the ARC under LD. We conclude that photoperiod-induced increase in VFI correlates with expression of NPY, but not with expression of genes for other putative orexigenic peptides. Ob-Rb gene expression is regulated by photoperiod.

seasonality; sheep; leptin

SPECIES LIVING in temperate and cold climates express profound seasonal cycles in food intake and body weight. This includes grazing ungulates, hibernators, and carnivores adapted to environments with food abundance in summer and food scarcity in winter. Comprehensive information on body weight cycles has been presented for domesticated sheep (1, 17, 20, 61), red deer (33, 73), reindeer (64), Siberian hamster (61, 87), woodchuck (21), mink (58), and black bear (44). In the long-lived species, it is suggested that the seasonal appetite cycle is generated endogenously as a circannual rhythm, and the annual change in photoperiod is utilized as the predictive environment cue to time the cycle (55). In short-lived species, such as small rodents, the seasonal changes in voluntary food intake (VFI) and body weight appear to be induced more by changes in photoperiod and nutrition (61). In general, exposure to long-day photoperiods (LD) stimulates VFI and weight gain, while exposure to short-day photoperiods (SD) has the reverse effect (54).

Photoperiodically induced changes in the VFI and body weight of sheep appear to depend on the presence of the mediobasal hypothalamic region, which includes the arcuate nucleus (ARC) (54). In particular, studies of ovariectomized female sheep living outdoors indicate changes in the expression of the gene for the orexigenic peptide neuropeptide Y (NPY) and NPY peptide levels in the ARC that correlate with seasonal changes in VFI (6, 20). Peak levels of NPY gene expression occurred in summer when VFI was increased, consistent with an orexigenic role for NPY. In another study, which used castrated Soay rams given constant-release estrogen implants, higher levels of prepro-orexin (ppORX) gene expression were found in the lateral hypothalamic area (LHA) of the hypothalamus under artificial SD compared with LD (3). Because orexins A and B are regarded as orexigenic peptides, and VFI is reduced under SD, it is not clear how orexins contribute to the seasonal regulation of VFI.

Reddy et al. (72) examined expression of NPY, pro-opiomelanocortin (POMC), and ppORX genes in the hypothalamus of the Siberian hamster and found no photoperiodically induced changes, despite changes in body weight. On the other hand, Mercer et al. (63) found that POMC gene expression decreased and agouti-related peptide (AGRP) gene expression increased in Siberian hamsters under SD, associated with reduced VFI and body weight. It is difficult to speculate as to how changes in POMC gene expression relate to the control of VFI, because one POMC-derived peptide

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[α-melanocyte-stimulating hormone (α-MSH)] is regarded as inhibitory, whereas another (β-endorphin) is regarded as stimulatory (67). It is possible that leptin regulates differential processing of the POMC precursor by influencing expression of the relevant enzymes. Because AGRP stimulates VFI (69), the SD-induced increase in expression of the gene that encodes this peptide is not consistent with the observed decrease in VFI under the winter photoperiod. Studies in the rodent model, therefore, do not provide a clear consensus view of which appetite-regulating peptides might mediate the effects of photoperiod to control VFI.

An important question is whether changes in the expression of genes for appetite-regulating peptides are a cause or a consequence of photoperiod-induced or seasonal changes in VFI. The regulation of VFI is under the control of a number of neuronal systems within the brain (67, 78), and long-term changes in nutrition affect expression of genes for many different appetite-regulating peptides. For example, diet-induced changes in body weight affect the level of expression of genes for NPY, AGRP, pro-enkephalin (ENK), somatostatin (SRIF), melanin-concentrating hormone (MCH), and cocaine- and amphetamine-related transcript (CART) in the hypothalamus of the ewe (42, 43, 48). In general, there is upregulation of orexigenic peptides (NPY, MCH, AGRP) in animals that have been reduced in weight by food restriction, with variable effects on other peptide systems. We (20) have proposed the hypothesis that photoperiod-induced changes in NPY expression in the ARC are of central importance in providing drive to seasonal shift in VFI, but the possible role of other appetite-regulating peptides has not been explored.

The aim of the present study was to quantify the level of expression of genes for appetite-regulating peptides in the hypothalamus of intact male sheep made hyperphagic by exposure to LD and hypophagic by exposure to SD. Rams of the Soay breed were used because they express a very marked photoperiod-induced cycle in VFI and body weight (54). Expression of genes for NPY, AGRP, ppORX, POMC, and MCH was quantified by in situ hybridization.

Because leptin has been identified as a factor regulating VFI and energy homeostasis (34, 90), we also tested the hypothesis that photoperiod-induced alterations in expression of genes for appetite-regulating peptides within the brain might be due to alterations in the level of expression of the leptin receptor. The signaling form of the leptin receptor (Ob-Rb) is found in all of the cell types that we have examined in the present study (49). Plasma leptin levels are highly correlated with white fat mass in sheep as in other species (11, 23), and Ob-Rb gene expression in the hypothalamus is reduced in Siberian hamsters under SD when blood leptin concentrations are decreased (63). In the sheep, an increase in the level of leptin receptor gene expression has been reported in hypothalamus of food-restricted females (25).

EXPERIMENTAL PROCEDURE

Ethics statement. These experiments were conducted under a Project License issued by the United Kingdom Home Office in accordance with the Animals Act (Scientific Procedures) of 1986. The work was also conducted in accordance with the "Guiding Principles for Research Involving Animals and Human Beings" of the American Physiological Society.

Animals. Adult rams of the Soay breed of feral sheep that express pronounced photoperiod-induced cycles of a variety of factors including food intake were used in this study (54). The animals were 1 yr old at the commencement of the study and weighed 18–23 kg. They were housed permanently in light-controlled rooms and individually penned with visual and tactile contact between neighbors. The animals were fed an ad libitum diet of commercial dried grass pellets (Vitagrass, Vitagrass Farms, Cumbria, UK) with hay and water ad libitum. Two similar groups of eight rams were housed in adjacent rooms and exposed to alternating 8- to 18-wk periods of long-day (LD; 16:8-h light-dark photoperiod) or short-day (SD; 8:16-h light-dark photoperiod) to differentially entrain their seasonal VFI cycle during a period of 42 wk. Physiological characteristics were measured on all eight animals, but only five animals from each group were randomly selected for in situ hybridization studies. The LD group received the sequence LD (12 wk), SD (12 wk), and LD and were killed 18 wk into LD (high VFI), and the SD group received the sequence SD (8 wk), LD (16 wk), and SD and were killed 18 wk into SD (low VFI). These lighting regimens were designed to cause a phase reversal in the timing of the VFI cycle and thus allow the two groups of animals to be killed at the same time but under contrasting photoperiods (and VFI status). Light intensity was ~160 lx at the animal's eye level. The time of lights-on was constant (0800), and the adjustments in photoperiod were achieved by abruptly changing the time of lights out by 8 h. Ambient temperature in the rooms was maintained between 10 and 20°C. To record changes in hormone levels, a blood sample was taken between 1000 and 1200 by jugular venipuncture once per week, 2–4 h after lights on and feeding. The blood samples were heparinized, and the plasma was separated by centrifugation within 30 min and stored at −20°C as three equal aliquots to avoid repeated refreezing for the multiple assays. Every week VFI was measured over a period of 24 h by providing 2 kg of the standard dried grass pellets and subtracting the weight of food remaining at the same time the following day. The food hoppers were designed to minimize food wastage. Every 2 wk, testis diameter was measured through the scrotum using calipers, and every 4 wk, the animals were weighed using a large animal cage balance. The animals were fully habituated to the artificial environment and to handling. The sheep were killed with an overdose of pentobarbital sodium (Euthatal, Rhone Merieux, Essex, UK). The brains were perfused with 4% paraformaldehyde and processed for in situ hybridization histochemistry as previously described (43). At autopsy, the total weight of abdominal fat was weighed, and the weight of the rumen/reticulum, abomasum, and intestines was recorded. Subtraction of the latter from the total body weight gave an indication of the extent to which a difference in body weight was due to a difference in stomach content (as a result of difference in VFI).

Cloning of ovine ppORX and ovine leptin receptor by RT-PCR. Total RNA was extracted from adult ovine lateral hypothalamus for ppORX and entire ovine hypothalamus tissue for Ob-Rb using the guanidine thiocyanate method. First-strand cDNA was synthesized using AMV-RT and oli-

go(dT) (Roche Applied Science, Indianapolis, IN). The RT reaction (with 1 μg of tRNA) was incubated at 42°C for 90 min followed by 95°C for 2 min in a final volume of 19 μl. For ppORX, the cDNA was used as a template for PCR with primers designed against the sequence from the human mRNA (GenBank: AF041240) and the rat mRNA (GenBank: AF041241). The primers used were 5′-hORX-138 (GCT GCT GCT RTC GCT GCC GCC) and 3′-hORX-322 (GCC TGM AGG AGG CGC TGC AGC CG). For Ob-Rb, primers were designed against the sequence from the ovine mRNA (Gen-Bank: OAU62124). The primers used were 5′-oLR-110 (GGA AAA ATA AAG ATG AGA TGG TG), and 3′-oLR-468 (GAA TGG AAG GTG TGG TGA A) (GIBCO BRL, New York). The PCR conditions for ppORX were 95°C for 5 min; 95°C for 60 s, 63°C for 90 s, 72°C for 90 s, for 40 cycles; and then 72°C for 5 min. The PCR conditions for Ob-Rb were 95°C for 5 min; 95°C for 60 s, 62°C for 90 s, 72°C for 90 s, for 40 cycles; and then 72°C for 5 min. The PCR products were then ligated into vector pGEMT-easy (Promega, Annandale, New South Wales, Australia) and transformed into XL1-Blue cells. Colonies were screened by blue/white and ampicillin selection, and plasmids from resultant cultures were isolated with Gene Works BRESApure plasmid maxi kit. The clones were subjected to restriction analysis (with *Eco*RI) to check for the expected 207-bp insertion for ppORX and the 377-bp insertion for Ob-Rb. Both fragments were sequenced. The 207-bp DNA fragment was found to have a high homology (94%) with the human ppORX sequence. The 377-bp DNA fragment was found to be homologous with the published ovine leptin receptor sequence (25).

In situ hybridization. Frozen 20-μm sections were cut using a cryostat and stored in a 2% paraformaldehyde/cryoprotectant solution at −0°C. For NPY and POMC, sections were taken from each animal to represent the rostral, medial, and caudal regions of the ARC. For AGRP, expression was very low in the SD animals, and statistical analysis could not be performed; expression was only measured in the medial and caudal subdivisions of the nucleus. For MCH and ppORX, two sections per animal were taken to include LHA, perifornical area (PFA), and dorsomedial hypothalamus (DMH). After in situ hybridization, one section was selected from each animal to best represent the region of interest, and image analysis was performed on this section. To examine Ob-Rb expression, sections were taken to include the medial ARC and the ventromedial nucleus (VMH) (found in the same section), both of which were analyzed. Expression for Ob-Rb was weak in other hypothalamic areas, and image analysis was not done.

For in situ hybridization, the sections were mounted onto Super Frost Plus slides (Menzel-Glaser, Braunschweig, Germany) and dried at room temperature overnight. In situ hybridization was performed using ^{35}S-dUTP-labeled (Amersham Pharmacia Biotech, Sydney, Australia) riboprobes following the method of Simmons et al. (80). The cDNA and plasmid inserts used were a 511-bp rat NPY insert in pGem 7Z, a 184-bp rat AGRP insert in pBKS$^-$, a 400-bp rat MCH insert in PCRII, and a 400-bp ovine POMC insert in PBSSK.

All cRNA riboprobes were synthesized using a Gemini system II kit (Promega Annandale, New South Wales, Australia). Hybridization was carried out at 53°C in a humid chamber for a minimum of 18 h, and slides were then treated with a series of posthybridization washes. The hybridization signal was detected using a Molecular Dynamics Storm PhosphorImager (Amersham Phamacia Biotech, Buckinghamshire, UK). The slides were exposed to a multipurpose Storage Phosphor Screen at room temperature for 1–3 days. Slides were dipped in Ilford K5 photographic emulsion (Ilford

Australia, Mount Waverly, Victoria, Australia) and exposed at 4°C for 7 days to 6 wk depending on the probe. For POMC and MCH, the slides were exposed on emulsion for 7 days. For NPY, exposure was 11 days; for AGRP, exposure was for 17 days; for ppORX, exposure was 9 days; and for Ob-Rb, exposure was 6 wk. The slides were developed using Ilford Phenisol developer, stop bath, and Hypam Fixer and then counterstained with 1% cresyl violet, dehydrated, and placed under a coverslip using DPX.

Radioimmunoassays. The concentrations of follicle-stimulating hormone (FSH) and prolactin were measured in the weekly blood samples collected from the rams using routine assays validated for sheep plasma for FSH (60) and prolactin (59). The FSH assay had a lower limit of detection of 0.2 μg/l NIDDK-FSH-RP2, and intra- and interassay coefficents of variation of 9.2 and 10.2%, respectively. The corresponding values for the prolactin assay were 0.4 μg/l NIH-PRL-S13, 6.5 and 9.8%, respectively. Concentrations of testosterone were measured using a method not involving chromatography and modified for an iodinated tracer (79). Plasma leptin concentration was determined in duplicate on 100-μl aliquots of weekly samples taken during the last 4 wk of the experiment using a previously described disequilibrium, double-antibody, ovine-specific RIA (23). The intra- and interassay coefficients of variation were 8 and 9%, respectively.

Assay of metabolic indicators. The plasma metabolites were determined enzymatically (31) by using an ELAN multianalyzer (Merck-Clevenot SA, Nogent-sur-Marne, France). Glucose concentration was determined by the glucose oxidase method (bioMérieux, Marcy-l'Etoile, France; Glucose RTU, no. 61 269). 3-Hydroxybutyrate levels were determined according to Ref. 8. Nonesterified fatty acid (NEFA) concentration was determined by the acyl-CoA synthetase method (Oxoid, Dardilly, France; NEFA C Wako, no. 46551). Acetate concentration was determined by the acetyl-CoA synthetase method (Roche, R-Biopharm, Darmstadt, Germany; acetic acid UV method, no. 0–148–261). Urea concentration was determined by the urease method (bioMérieux, Marcy-l'Etoile, France; urée cinétique UV 250, no. 61 974). Phospholipid concentration was assayed by the phospholipase D and choline oxidase method (bioMérieux, Marcy-l'Etoile, France; phospholipides enzymatiques PAP 150, no. 61 491).

Analysis of in situ hybridization data. Emulsion-dipped slides were analyzed at a cellular level by counting the number of labeled cells and silver grains per cell. Between 20 and 50 cells per section were randomly selected and analyzed by counting the number of silver grains. Silver grain counting was performed at ×400 magnification using a microcomputer imaging device (MCID) MI system from Imaging Research (Brock University, St. Catherines, Ontario, Canada). The number of labeled cells was counted at ×20 magnification.

Statistics. Homogeneity of variance was checked and statistical comparisons were made using one-way ANOVA. Post hoc testing for differences between means used the method of least significant differences.

RESULTS

Animal measurements. Figure 1 shows the pattern of change in VFI and body weight in the two groups of animals in the transitions through LD and SD photoperiods over the 42-wk experiment. In the SD group, the animals showed an initial increase in VFI and body weight on LD. VFI was maintained at a high level and then began to decline progressively after 7 wk after transfer to SD (at 31 wk). The animals were clearly
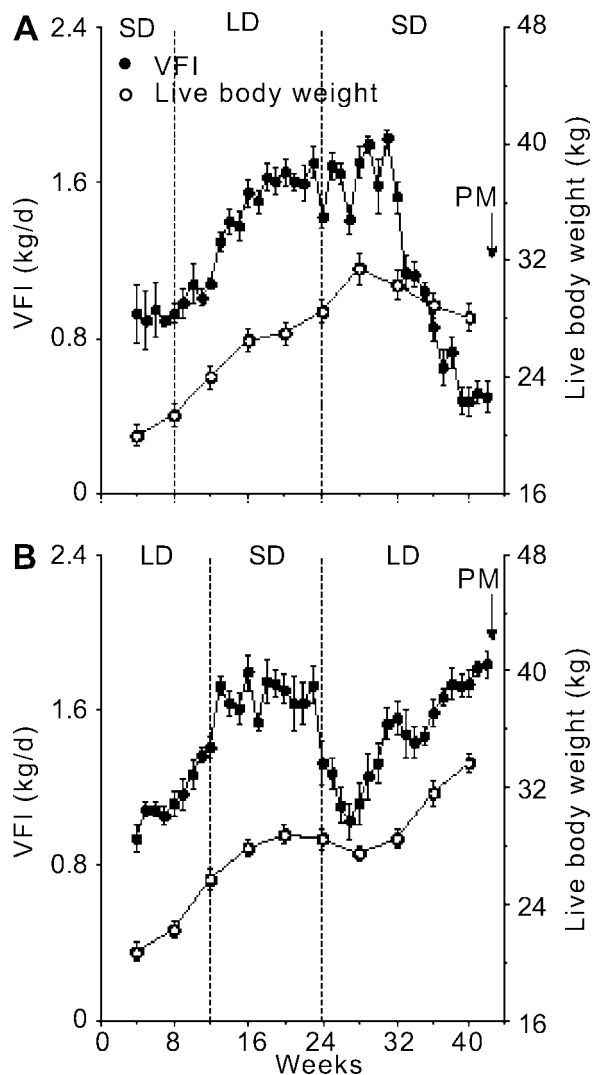
Fig. 1. Patterns of change in voluntary food intake (VFI) and body weight (means ± SE) in 2 groups of Soay rams during transitions through long-day (LD) and short-day (SD) entrainment schedule. The SD group (A) was killed after 18 wk on SD, and the LD group (B) was killed after 18 wk on LD. The different pretreatment photoperiod regimen for the 2 groups was designed to induce a phase reversal in the timing of the VFI cycle within the 42-wk experiment. PM, postmortem.

hypophagic, and body weight was declining when they were killed at 18 wk into SD. In the LD group, VFI and body weight initially increased under LD, and then maintained under SD. After the final transfer to LD, VFI and body weight decreased and then increased progressively. The animals were hyperphagic, and body weight was increasing when they were killed at 18 wk into LD.

Table 1 presents the final weights and the metabolic parameters for the Soay rams killed under LD and SD photoperiods. The LD animals were significantly ($P < 0.005$) heavier than the SD animals. The LD animals had significantly ($P < 0.0003$) greater gut weight as a result of their higher ($P < 0.0009$) VFI. When the weight of the gut was subtracted from the total body weight, there was no difference in the residual weight

of the animals in the two groups. The amount of abdominal fat and the plasma leptin levels of the two groups were similar. When expressed as percentage of body weight or percentage of body weight less gutfill, the amount of abdominal fat was similar in the two groups of animals (data not shown).

The metabolic profile of the LD animals indicated that they were deriving a high number of calories from dietary sources and not mobilizing fat stores. Thus the LD animals had lower ($P < 0.05$) plasma NEFA levels compared with the SD animals. Consistent with a high absorption rate from the gut, the LD animals had higher urea, phospholipid, 3-hydroxybutyrate, and acetate levels and were maintaining a slightly higher ($P < 0.05$) plasma glucose level.

Figure 2 shows the plasma testosterone, prolactin, and FSH concentrations for the two groups of animals as well as the testis diameter. In the SD group, plasma prolactin concentrations were initially low during SD, rose with the switch to LD, and then fell with the final switch to SD. Plasma concentrations of FSH increased markedly after this switch to SD associated with growth and activation of the testes. The animals were just past the peak in the testicular cycle, with large testes and high plasma concentrations of testosterone, when killed at 18 wk into SD. In the LD group, plasma prolactin concentrations initially increased under LD, declined under SD, and rose again during the final period of LD (Fig. 2). Plasma FSH concentrations were low during the initial period of LD, rose to a maximum under SD, and then declined under LD with corresponding changes in testis diameter. The animals were just past the nadir of the testicular cycle, with small testes low plasma concentrations of testosterone, when killed at 18 wk into LD. At the time the animals were killed, plasma testosterone levels were 13.8 ± 1.5 ng/ml in SD and 1.6 ± 0.4 ng/ml in LD ($P < 0.0001$), plasma prolactin levels were 8.7 ± 1.3 ng/ml in SD and 54.6 ± 9.2 ng/ml in LD ($P < 0.0002$), and plasma FSH

Table 1. *Body weight, gut weights, abdominal fat weights, and metabolic parameters in Soay rams on LD and SD photoperiods*

Group	SD	LD	Significance
Body weight, kg	28.65 ± 0.99	35.65 ± 0.99	0.005
Gut weight, kg*	5.9 ± 0.7	11.2 ± 0.04	0.0003
Body weight less gut weight, kg	22.4 ± 1.0	25.3 ± 0.7	NS
Total abdominal fat, g†	708 ± 54.1	896 ± 82.9	NS
Voluntary food intake, kg	0.6 ± 0.16	1.83 ± 0.06	0.0009
Leptin, ng/ml	5.1 ± 0.2	5.9 ± 0.6	NS
NEFA, mmol/l	0.18 ± 0.03	0.09 ± 0.007	0.05
Glucose, g/l	0.57 ± 0.01	0.64 ± 0.02	0.05
3-Hydroxybutyrate, mmol/l	0.38 ± 0.02	0.51 ± 0.03	0.01
Acetate, mg/l	45.6 ± 7.0	80.1 ± 7.7	0.01
Urea, g/l	0.32 ± 0.02	0.47 ± 0.007	0.0001
Phospholipids, mmol/l	0.70 ± 0.03	0.84 ± 0.03	0.007

Data are means ± SE. *Weight of the rumen, reticulum, omasum abomasum, and intestines. †Weight of omental fat and kidney fat. LD, long day; SD, short day; NEFA, nonesterified fatty acids.
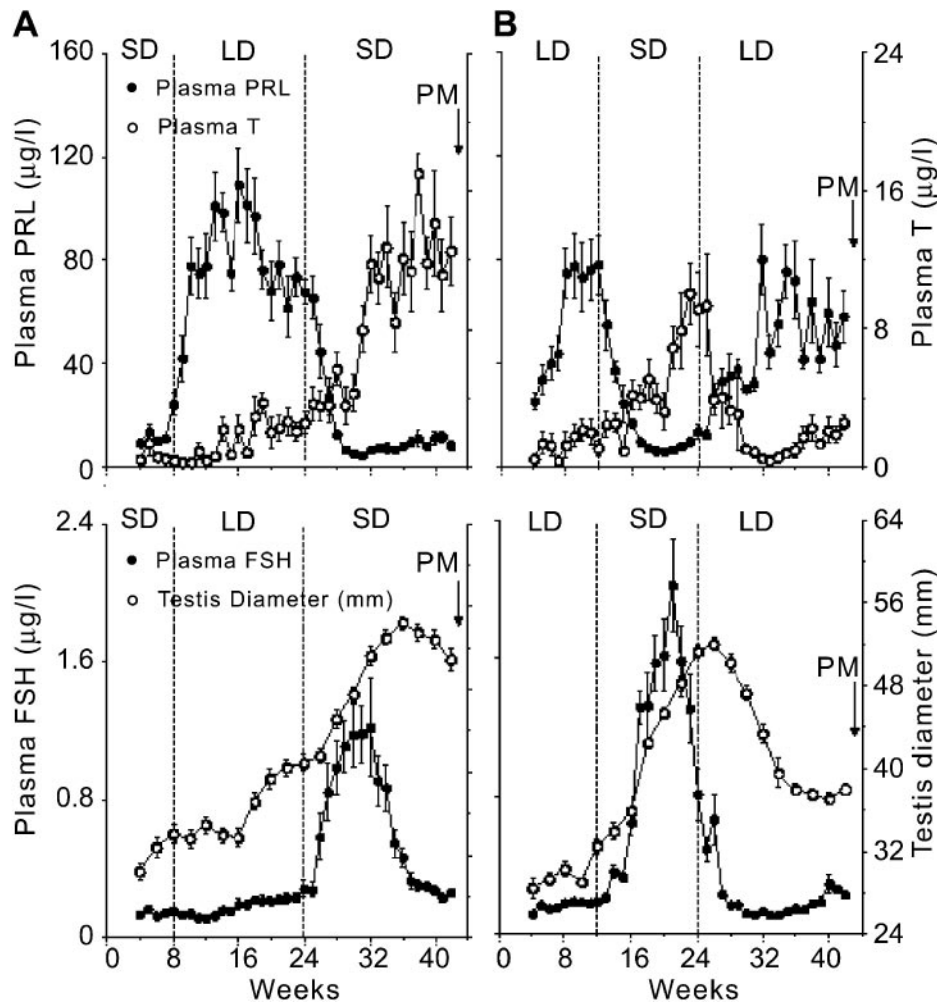
Fig. 2. Mean ± SE plasma testosterone (T), prolactin (PRL), and follicle-stimulating hormone (FSH) concentrations, and mean ± SE testis diameter for the SD (*A*) and LD groups (*B*) during transitions through LD and SD photoperiods.

testosterone levels were 0.5 ± 0.05 ng/ml in SD and 0.4 ± 0.05 ng/ml in LD (NS). Testis diameter was 50.9 ± 1.1 mm in SD and 40.4 ± 0.6 mm in LD ($P < 0.0001$).

In situ hybridization. Figures 3 and 4 give examples of in situ hybridization for NPY, POMC, and AGRP in the ARC, and quantitative image analysis data for NPY and POMC are presented in Fig. 5. The distribution of cells expressing genes for these neuropeptides was as previously described (20, 40, 42, 43). In the caudal region of the ARC, the number of cells expressing NPY mRNA was higher ($P < 0.01$) in the LD animals than in SD animals, but this difference was not seen in the mid- or rostral regions of the ARC. Expression of NPY mRNA/cell was higher ($P < 0.01$) throughout the ARC in the LD animals (Fig. 5).

There was a lower ($P < 0.05$) number of cells hybridized with the POMC probe in the midregion of the ARC in the LD group, with no differences in the rostral or caudal regions. The level of POMC expression/cell was similar throughout the ARC (Fig. 5). AGRP gene expression was strongly upregulated in LD animals (Fig. 3), and expression was so low in the SD animals that a statistical analysis was not performed.

Figure 6 shows low-power images of ppORX and MCH gene expression, which was distributed throughout the DMH, PFA, and LHA. In the regions of the PFA and the DMH, MCH gene expression/cell was lower ($P < 0.05$ for PFA and $P < 0.02$ for DMH) in the LD group, but there was no difference between the groups in the LHA (Figs. 7 and 8). There was no difference between the groups in the number of cells expressing MCH mRNA (Fig. 8). Within these regions of the hypothalamus, there was no difference between the LD and the SD groups in the expression of mRNA for ppORX (Figs. 6 and 7).

Figure 9 presents examples of Ob-Rb gene expression in the ARC and the ventromedial nucleus of LD and SD animals. There was no difference in the number of cells expressing Ob-Rb (data not shown). LD animals showed a higher level of Ob-Rb gene expression per cell in the ARC (360.3 ± 18.5 vs. 216.1 ± 23.3; $P < 0.001$), but there was no significant difference in the level of expression/cell in the ventromedial nucleus (SD 315.9 ± 23.7 vs. LD 389.5 ± 34.9).

Using pooled NPY data for the number of cells labeled in the SD and LD groups, there was a significant ($P < 0.05$) correlation ($R^2 = 0.68$) with VFI. VFI ex-
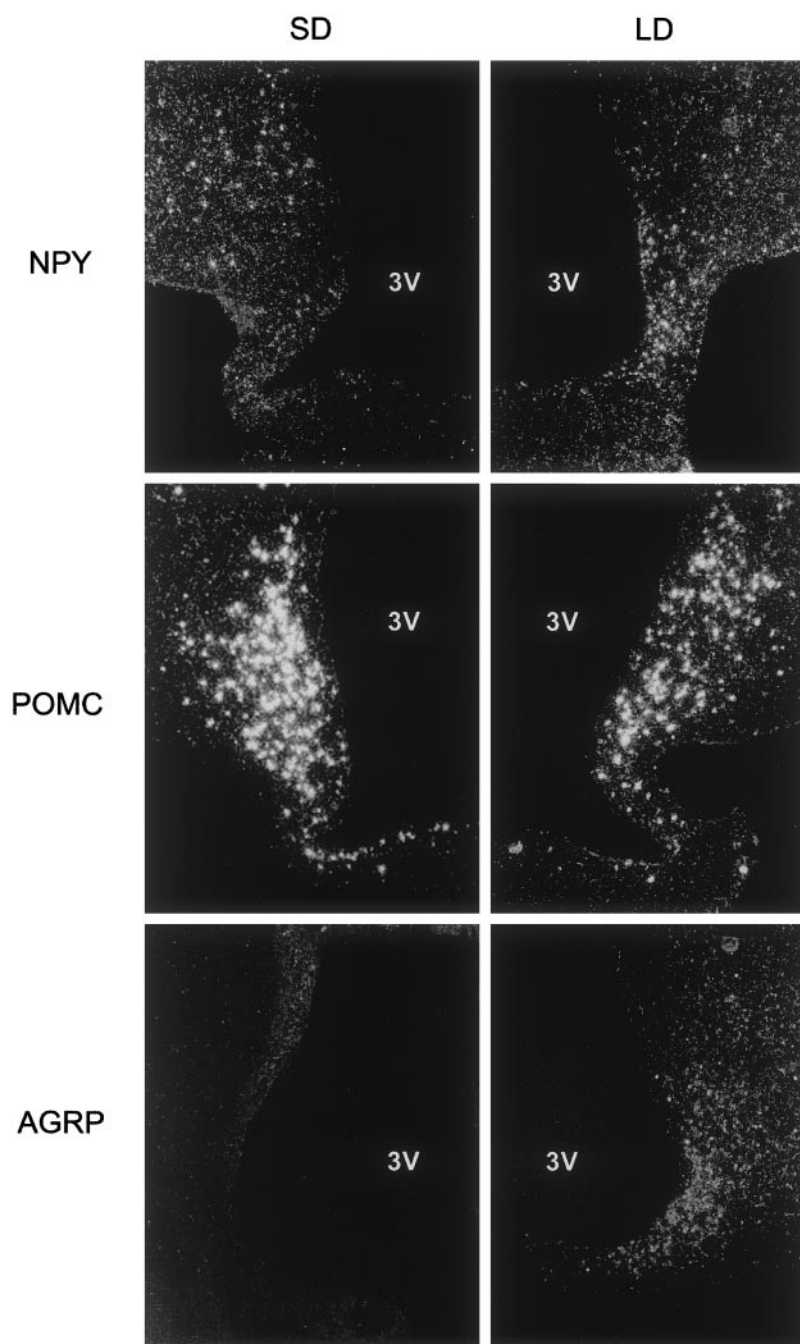
Fig. 3. Representative dark-field micrographs (×2 magnification) of neuropeptide Y (NPY), pro-opiomelanocortin (POMC), and agouti-related peptide (AGRP)-labeled cells in the arcuate nucleus (ARC) of Soay rams on LD and SD photoperiods. 3V, 3rd ventricle.

pression was not correlated with the expression of any of the other genes examined.

DISCUSSION

This detailed study, conducted under controlled photoperiod, shows that expression of the genes for NPY, AGRP, and Ob-Rb are upregulated under long days, in association with an increase in appetite. Expression of the gene for POMC and MCH is reduced under LD, and expression of the gene for ppORX does not change with photoperiod. Based on the known effects of the peptide products of these genes on appetite, upregulation of NPY and AGRP gene expression is most likely to be causally linked to the LD-induced increase in VFI in rams. On the other hand, the observed increase in expression of Ob-Rb during LD is contrary to the notion that higher sensitivity to leptin would be associated with reduced VFI, because the animals were eating more under this photoperiod.

This study of gonadal-intact rams provides data on gene expression in the brain under carefully controlled conditions, but a variety of factors warrant consideration. The experimental design employed opposite alternating periods of LD and SD to differentially entrain the long-term seasonal cycles in the two experimental groups. This was considered the most
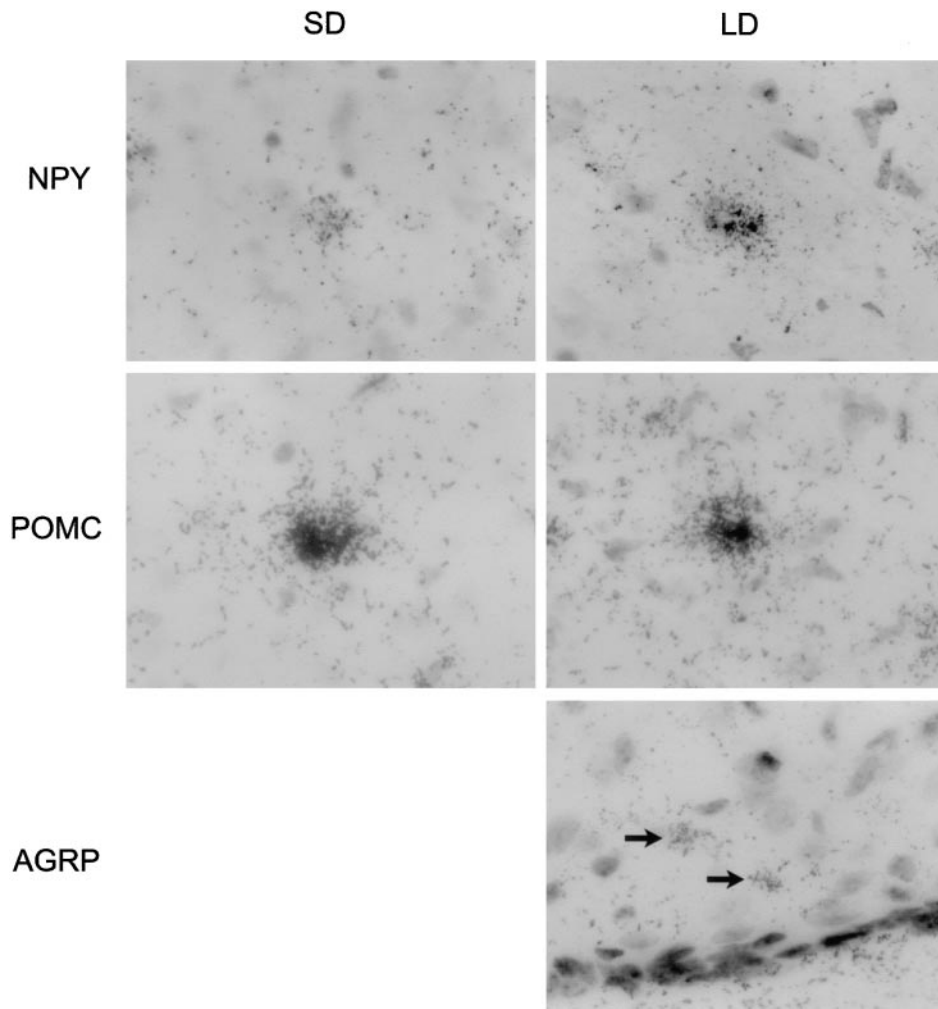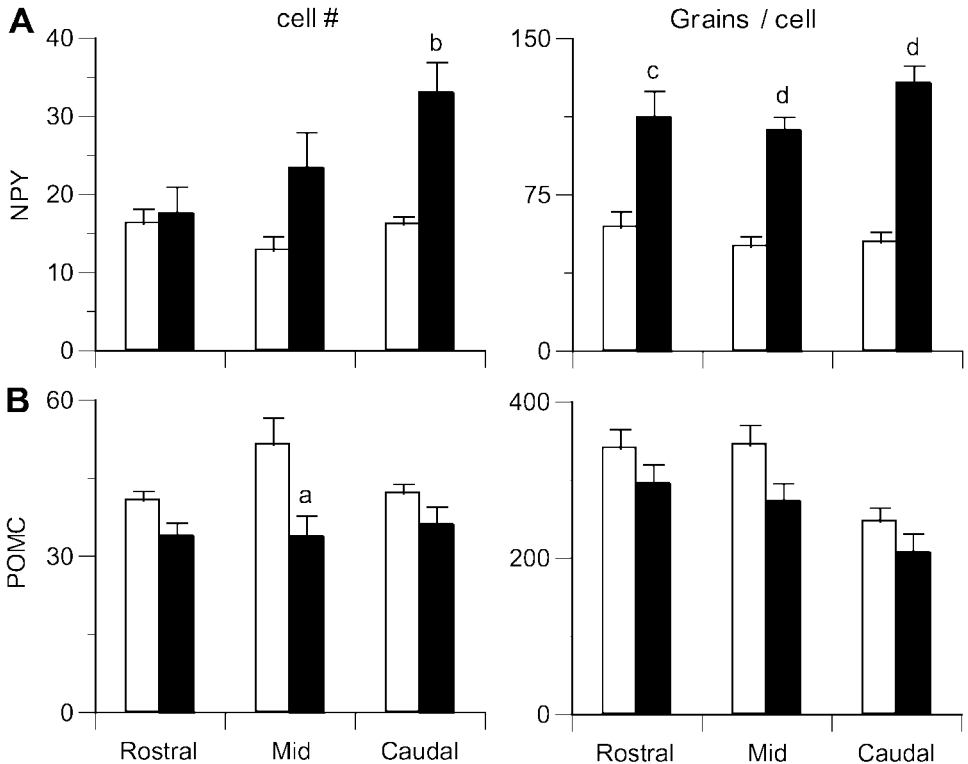
Fig. 4. Examples of in situ hybridization (×40 magnification) for NPY and POMC in the ARC of Soay rams on LD and SD and AGRP in rams on LD. Cells expressing AGRP were not detectable in the SD animals. Arrows, cells expressing AGRP.

effective way of demonstrating an effect of photoperiod on the control of VFI, because the animals could be killed at the same time, but under contrasting photoperiods and in very different physiological states. The schedule for the SD group was essentially similar to that occurring naturally outdoors, while the schedule for the LD group phase-advanced the VFI cycle by early exposure to LD followed by SD. In this group, the final switch from SD to LD was very effective at reactivating an increase in VFI. Thus, when the animals were killed, one group was hyperphagic under LD, while the other group was hypophagic under SD, eating only 25% as much food. We chose to study gonadal-intact animals, even though there would be confounding effects of gonadal steroids and photoperiod. This was done to examine the photoperiodically induced changes that occur in the complete organism, as a first step toward understanding changes in gene expression that might relate to VFI and/or reproductive (or other) function.

The artificial lighting regimens had somewhat different effects on the various endocrine systems, as expected from previous studies. Plasma prolactin concentrations were elevated on LD and suppressed on SD photoperiod, with no complex dynamics. This is consis-

tent with the view that photoperiodic history has minimal effect on seasonal patterns of prolactin secretion (38). It is known that photoperiod-induced changes in prolactin regulate seasonal patterns of pelage and horn growth (51), but it is unlikely that prolactin influences VFI, based on earlier studies (29). The photoperiodic responses of the reproductive axis of the animals in the present study were consistent with previous work on this model and the ram in general (2,51,83). The minor decline in testis diameter at the end of the study indicates that the animals were becoming photorefractory as expected at 18 wk into SD. Reciprocal events were evident in the LD group where the gonadotropin/gonadal axis was beginning to reactivate at 18 wk into LD. Despite these dynamics in the plasma concentrations of the reproductive hormones, the plasma testosterone levels were clearly very different in the SD and LD animals, reflecting the sexually active and inactive states. Such changes in reproductive status could influence VFI. Interestingly, in the SD group, the decrease in VFI at 7 wk after the switch from LD to SD was coincident with the large increase in plasma testosterone levels, whereas in the LD group the activation of VFI at 4 wk after the switch from SD to LD

Fig. 5. Mean ± SE of number of cells expressing NPY or POMC and mean ± SE of number of silver grains/cell in the rostral, medial (Mid), and caudal ARC of Soay rams on LD and SD. Open bars, SD; filled bars, LD. [a]$P = 0.026$ vs. SD. [b]$P = 0.015$ vs. SD. [c]$P < 0.01$ vs. SD. [d]$P < 0.001$ vs. SD.

occurred several weeks after the decline in plasma testosterone levels. The question as to how testosterone affects food intake has been studied in Siberian hamsters and rats, but the issue is not entirely resolved. Orchidectomy reduces VFI in rats, an effect reversed by testosterone treatment, although there are important time-dependent and dose-related effects (68). In male Siberian hamsters, testosterone treatment reversed the SD-induced decrease in VFI, but dihydrotestosterone treatment exaggerated the effects of SD (10). Further studies are required to ascertain the androgenic effect on VFI.

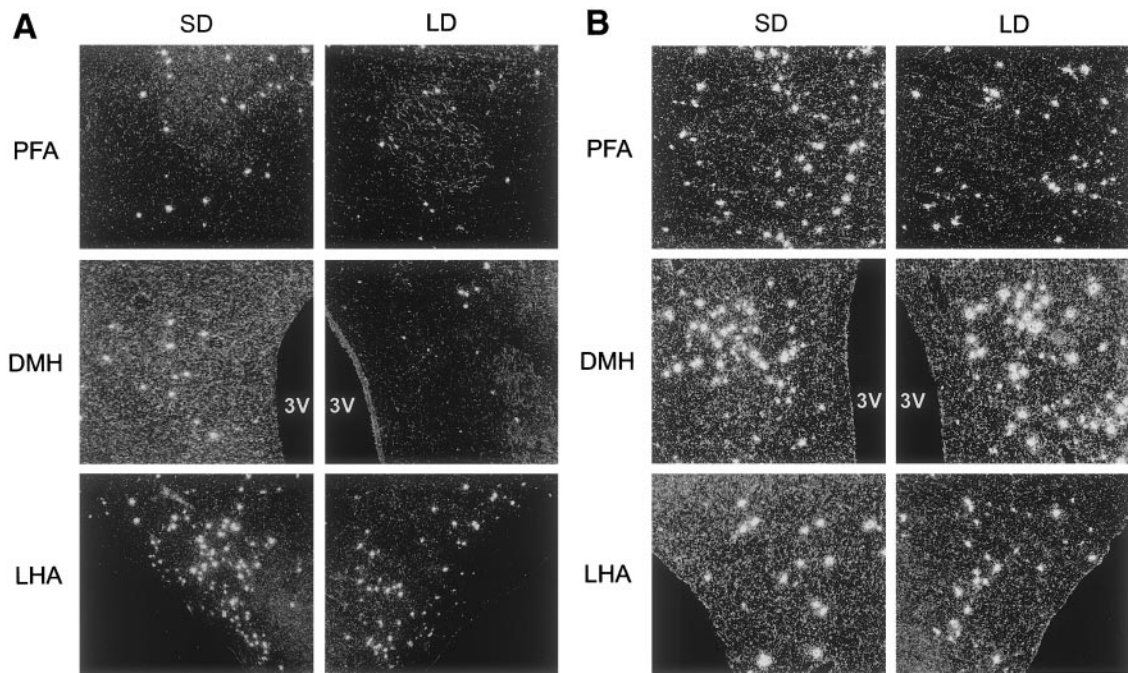Fig. 6. Representative dark-field micrographs (×2 magnification) of melanin-concentrating hormone (MCH)-labeled (*A*) and ppORX-labeled cells (*B*) in the perifornical area (PFA), dorsomedial hypothalamus (DMH), and lateral hypothalamic area (LHA).
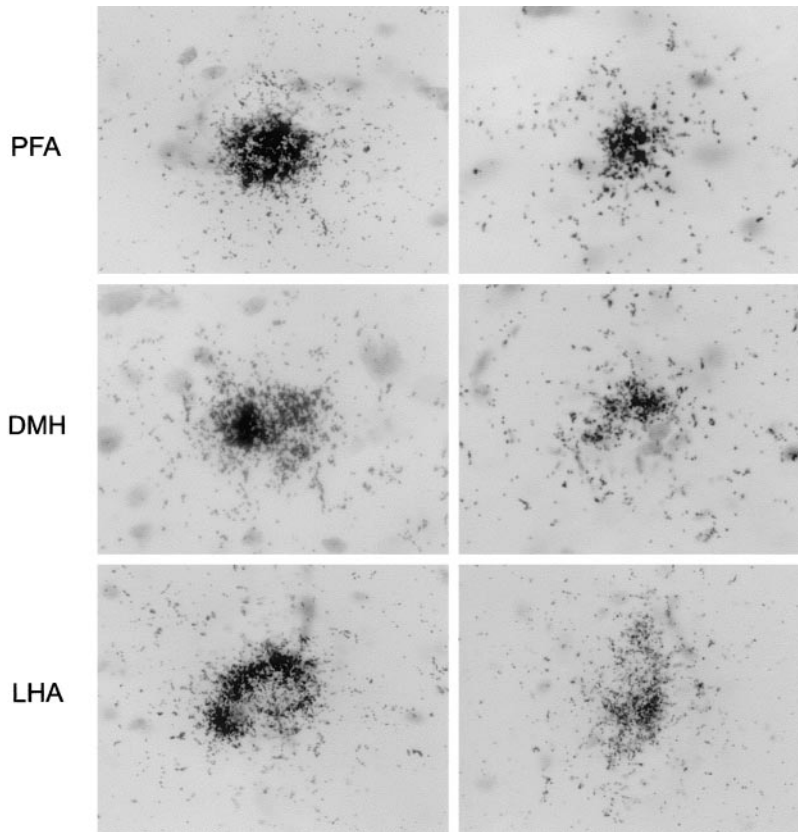
Fig. 7. Examples of in situ hybridization (×40 magnification) for MCH in the PFA, DMH, and the LHA.

Using the current paradigm of photoperiodic manipulation, we were able to obtain animals that had no significant difference in body weight after accounting for the gutfill. Adiposity was estimated from the absolute and relative weight of abdominal fat. The amount of abdominal fat was similar in the animals killed on LD and SD photoperiods, suggesting that the two groups of animals were similar in terms of adiposity and the plasma leptin values concur with this. Plasma leptin levels in sheep are correlated with adiposity (11, 23, 28). On the other hand, Marie et al. (57) reported that plasma leptin levels in Soay rams were increased more than twofold after 16 wk of LD conditions, in association with a 15% increase in body condition score (an indirect measure of adiposity) and a 28% increase in body weight (not corrected for gutfill). It is also possible that the slight increase in plasma testosterone levels in LD animals between *weeks 36* and *42* (i.e., when leptin was measured) could have inhibited leptin secretion by adipose tissue, because testosterone increases fat-free mass and reduces fat mass and plasma leptin levels, albeit in humans (4).

In contrast to the similarity between the groups in adiposity and plasma leptin levels, the metabolic profiles of the LD and SD animals were quite different. The lower levels of NEFA and free glycerol are consistent with lower rates of fat mobilization in the LD days, and the higher levels of glucose, acetate, phospholipids, and 3-hydroxybutyrate indicate derivation of energy from dietary sources. The higher plasma levels of urea could indicate protein excess, being derived from the diet, or reduced protein deposition or an amino acid imbalance. The metabolic profiles compare with those seen in underfed and well-fed animals (18) and indicate that the LD animals were in positive energy balance, whereas the SD animals may have been in negative energy balance at the time they were killed. The sexual state and plasma testosterone levels of the two different groups may, in part, explain these different metabolic conditions. Earlier work (52) showed that aggressive behavior toward animals in the neighboring pens is stimulated under SD conditions, perhaps increasing energy expenditure.

NPY is perhaps the most potent of the orexigenic peptides of the brain (50) and is strongly implicated as a central player in the regulation of food intake in a variety of species (50), including the sheep (65). The cells of the ARC are thought to be central to the regulation of VFI, and the NPY- and POMC-expressing cells of this region express a high level of leptin receptors in various species, including sheep (49, 88). NPY gene expression is also upregulated in chronically food-restricted sheep, suggesting an adaptive mechanism to increase hunger drive (42). On the other hand, central infusion of leptin into sheep reduces gene expression for NPY (40). In the present study, increased numbers of cells expressing the NPY gene were found in the caudal ARC in LD animals and increased expression of the NPY gene/cell was seen across the entire ARC in LD animals. Upregulation of NPY gene expression under LD is consistent with a role for this neuropeptide in mediating the photoperiodic effect on VFI. This is
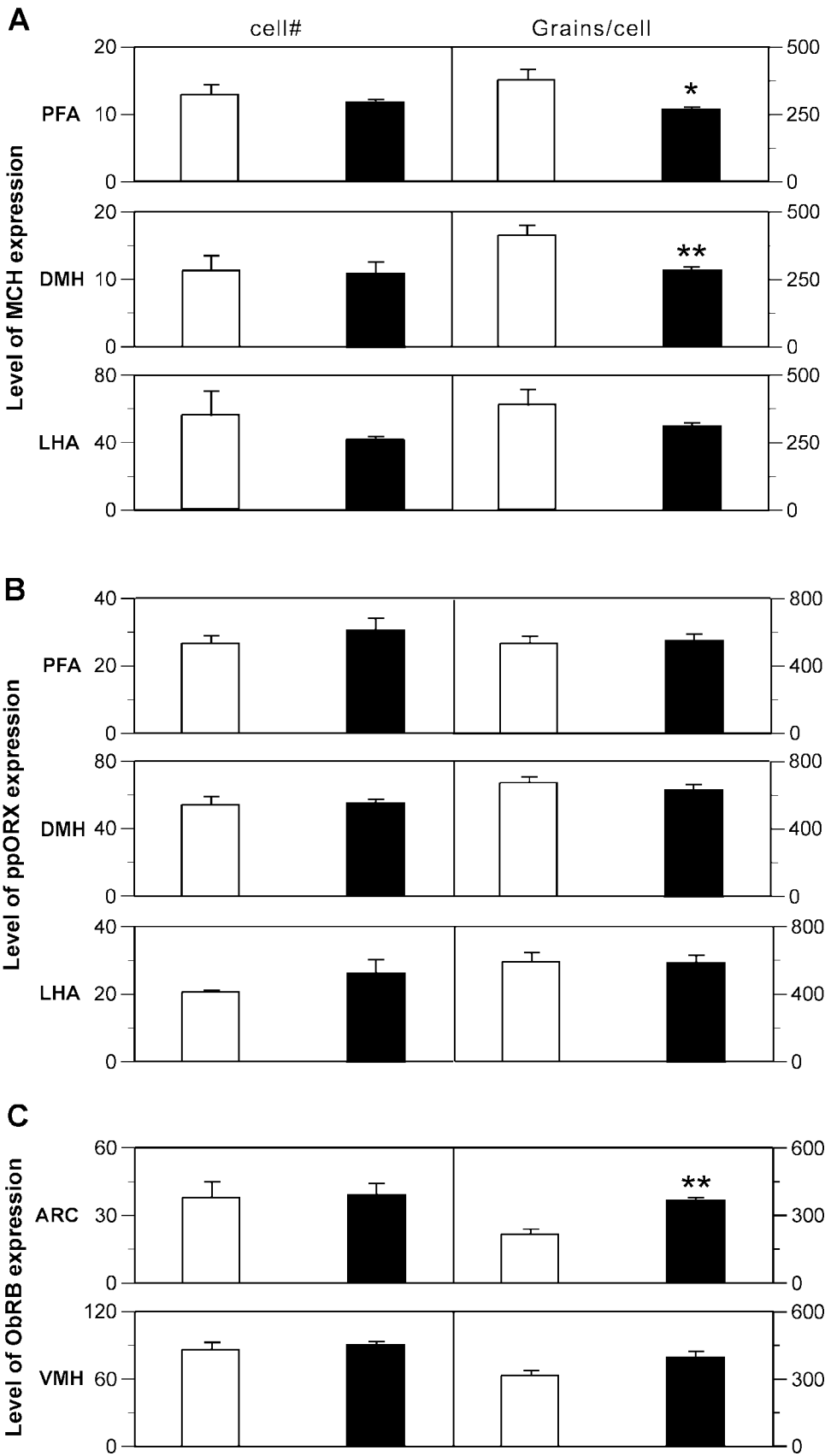
Fig. 8. Expression of MCH (*A*), prepro-orexin (ppORX; *B*), or Ob-Rb (*C*), showing mean ± SE of number of labeled cells, and mean ± SE of number of silver grains/cell in the relevant hypothalamic nuclei. Open bars, SD; filled bars, LD. VMH, ventromedial hypothalamus. *$P < 0.05$, **$P < 0.02$ vs. SD.
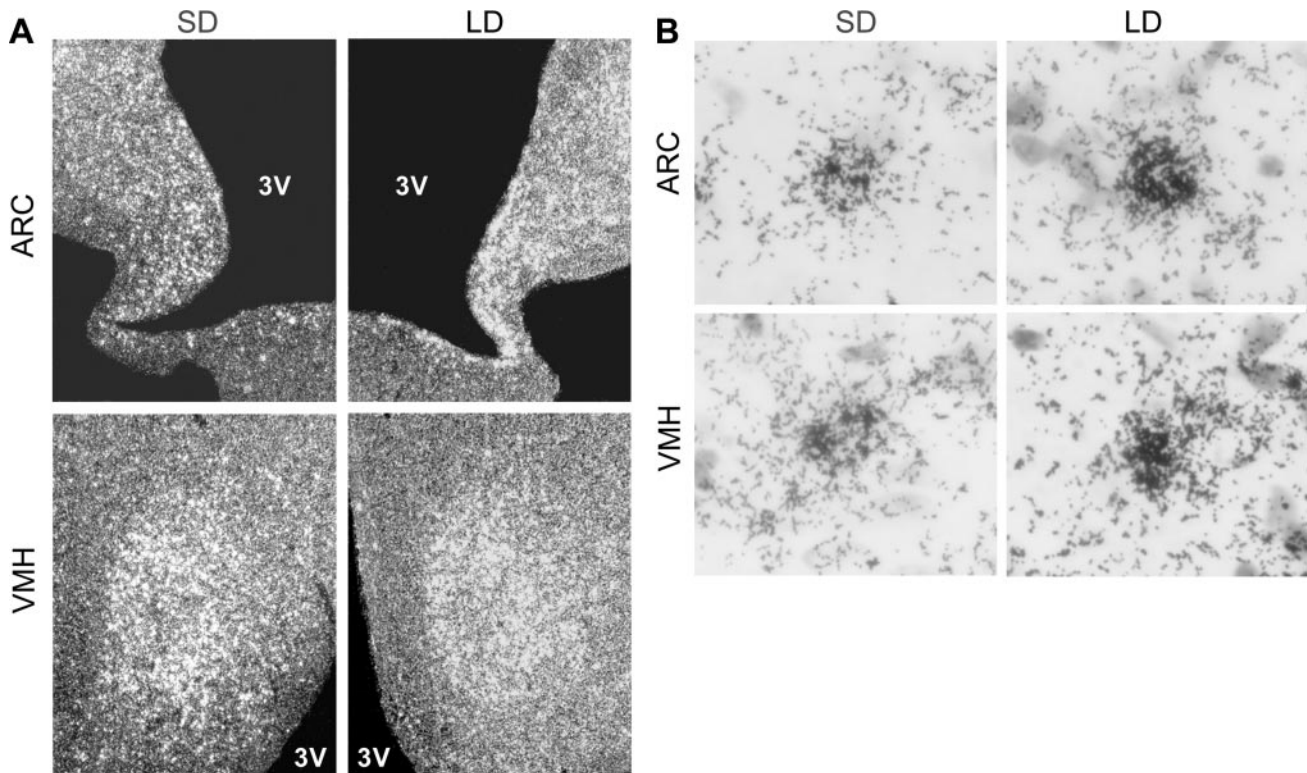
Fig. 9. Representative dark-field micrographs (*A*) (×2 magnification) and examples of in situ hybridization (*B*) (×40 magnification) for leptin receptor in the medial ARC and the VMH.

similar to the results observed in female sheep where expression increases under the influence of increasing daylength (6, 20) but contrasts with variable effects obtained in Siberian hamsters (62, 63, 72). The sheep differs from the hamster in a number of ways, however, that may be relevant to this species difference. First, hamsters are nocturnal and sheep are not. Second, as pointed out in the introduction, sheep use photoperiod to cue circannual rhythms, whereas shorter-lived species like hamsters have more short-term strategies. Third, hamsters breed under LD photoperiod, and sheep breed in response to SD. In this regard, the question of whether the effects of photoperiod on the expression of genes in the hypothalamus are related to reproductive function rather than VFI or other functions must be considered. For example, a subset of NPY neurons expresses the estrogen receptor (81), and NPY has regulatory effects on the gonadotropin-releasing hormone/gonadotropin axis (7, 71). In this respect, it is important to note that the animals of the present study were killed at a time when plasma testosterone levels were at least fivefold different, and future studies should consider the relevance of this difference to the regulation of food intake.

Expression of the gene for AGRP was strongly upregulated in the LD animals, suggesting that this may play a fundamental role in the stimulation of VFI in this group. AGRP colocalizes with NPY in the cells of the ARC (36) and is thought to be an antagonist of α-MSH at the MC-3R/4R melanocortin receptors (32, 69). By preventing the inhibitory action of α-MSH on VFI, AGRP may act as a stimulator of appetite (74). Projections of AGRP neurons (that also contain NPY) to other appetite-regulating cells of the hypothalamus in the rat and monkey have further implicated this peptide in regulation of VFI (13–15, 27, 30, 37, 46, 89), and the AGRP gene expression is upregulated in fasted rats and can be manipulated by leptin treatment (66, 89). AGRP gene expression is also increased by chronic food restriction in sheep (42). Upregulation of expression of the gene for AGRP under LD photoperiod is consistent with increased appetite drive in this group of animals. This result contrasts with that found in the hamster where expression was reduced under LD (when VFI is increased) (63).

We found that the number of cells expressing POMC was reduced in animals on LD photoperiod, but only in the medial division of the ARC, and the extent to which this might relate to changes in VFI is an important question. It is clear from studies in rodents and humans that the melanocortin system plays an important role in the central role in the regulation of VFI (9, 78), but we have consistently found no alterations in expression of the POMC gene with chronic food restriction or increased adiposity in sheep (42). Despite this, a high percentage of POMC-expressing cells contain Ob-Rb, suggesting regulation by leptin (49). The POMC precursor can be processed to produce α-MSH and β-endorphin (82), which respectively inhibit and stimulate VFI (5, 84). Given that there is dual production from the same gene product of peptides that either inhibit or stimulate food intake, it would seem unlikely

that genomic regulation would be an efficient means of regulation. On the other hand, the POMC precursor is processed by a number of enzymes including proconvertase I and II and carboxypeptidases (16), and these could be regulated to alter the ratio of production of α-MSH and β-endorphin. Further study is required to determine whether this might be the case. Hileman et al. (45) reported that POMC gene expression was lower in castrate rams treated with testosterone under LD conditions, which agrees with the current result. On the other hand, we (20) and others (81) have respectively found that POMC gene expression and immunocytochemically identified β-endorphin cells were fewer in the ARC of ewes during the nonbreeding season (increasing daylength synonymous with LD). Thus it would appear that in the sheep, there is a sex difference in the way that photoperiod regulates the expression of this gene.

Expression of the MCH gene increases with reduced body weight in ovariectomized ewes (40), consistent with the orexigenic function of this peptide. This is consistent with a generalized increased hunger drive in animals that have restricted food intake being manifest in upregulation of stimulators of VFI. It was surprising, therefore, to find that expression of the MCH gene was reduced under LD photoperiod in the animals of the present study. Various studies suggest that MCH stimulates the reproductive axis (19, 35), but others show an inhibitory effect (85). If the former is correct, then reduction of expression at the time of reproductive quiescence (LD) may provide a possible explanation for the present result. In other words, the expression of MCH may be more closely linked to reproductive function than to regulation of appetite.

The orexins are located in the LHA, a region that is important in the regulation of food intake sheep as in other species (3, 70). In addition to being orexigenic in the sheep (77), pigs (26), and other species (75, 76), the orexins have important roles in neuroendocrine function, arousal, and activity (22, 86). Intracerebroventricular injection of orexin has been shown to cause a stimulation of VFI in sheep within 2 h, although the effect is no longer apparent within 4 h (77). In this respect, the effect is different from that obtained with NPY, which causes a much longer-lasting effect (65). Information on the expression of the ppORX gene has been obtained from castrate Soay rams that were treated with estrogen and held under LD or SD photoperiod. In that study, ppORX expression was higher under SD when VFI is reduced (3), clearly not supporting a role as an orexigenic agent in this circumstance. Neither were changes in ppORX gene expression seen to accompany the changes in VFI that occurred with changing photoperiod in Siberian hamsters (63, 72). On the other hand, ppORX gene expression was not affected by a 4-day fast in sheep (3), which contrasts with data from the rat (76), but is similar to results obtained in Siberian hamsters (63, 72). It is possible that orexins play a role in the short-term regulation of feeding behavior, but not in long-term regulation maintenance of homeostasis. In the present study, we found that ppORX expression was similar under LD and SD photoperiod in three separate regions of the hypothalamus. This suggests that the orexin peptides do not play a substantive role in the photoperiodically driven change in VFI that occurs in Soay rams. The conclusion that ppORX expression is not strongly correlated with VFI in the sheep is also substantiated by the observation that no changes occur with diet-induced alteration in body weight or during the natural photoperiodic cycle of ovariectomized female sheep (47).

The signaling form of the leptin receptor (Ob-Rb) has been localized to NPY cell ovine ARC by in situ hybridization (88) and other cells of the hypothalamus by immunohistochemistry (49). A preliminary study (25) suggested that expression of the Ob-Rb gene is increased in food-restricted ovariectomized ewes. Such a metabolic state produces hunger, and it is difficult to develop a model that incorporates this alteration in Ob-Rb. In male Siberian hamsters (63), Ob-Rb expression was increased in the brain under LD photoperiod, which is a time of reduced VFI. Clearly, the results of the present study are opposite to this, in the sense that upregulation of Ob-Rb in the ARC under LD in the Soay ram is concomitant with a period of increased VFI. There is no clear explanation of this result. Leptin stimulates the reproductive axis in sheep (41), and this might be aided by upregulation of Ob-Rb, but the present result is contrary to this hypothetical notion (LD animals are sexually quiescent). On the other hand, the photoperiodic manipulation that we employed allowed escape from sexual inactivity under LD, and this might have been aided by upregulation of the Ob-Rb.

One obvious question is how the effects of altered photoperiod might lead to changes in expression of genes for NPY, AGRP, Ob-Rb, and POMC cells. In this respect, it is interesting to note that melatonin binding sites are found in the mediobasal hypothalamus/arcuate region of the ovine brain (39). Although there is no definitive information on the cell type that expresses these receptors, infusion into the mediobasal hypothalamus has significant effects on the reproductive axis (53, 56). Thus it is possible that melatonin can act directly on the NPY cells and regulate gene expression in the sheep, but this remains to be determined. It is particularly interesting to note that the number of NPY cells detected by in situ hybridization was increased in the caudal region of the ARC, because melatonin binding has been localized to the premammillary region (56). This coincidence could mean that this subset of cells is especially susceptible to regulation by melatonin, although the level of expression in NPY cells was increased across the ARC in the LD animals. Delavaud et al. (24) found that melatonin treatment of ewes for 69 days did not affect plasma leptin levels, but placement of microimplants of melatonin in the mediobasal hypothalamus of rams under LD can alter the timing of the body weight cycle (53), suggesting that melatonin plays a role in the regulation of food intake.

In conclusion, the present data present strong evidence that photoperiodically driven changes in VFI are

related to upregulation of expression of genes for NPY and AGRP. Expression of ppORX does not change with changes in photoperiod in the gonad-intact ram. Differences in the expression of genes for POMC, MCH, and Ob-Rb are also seen on LD and SD photoperiods, but it is difficult to incorporate these into a model that would explain the concomitant changes in VFI. It is possible that the expression of these genes might relate to the regulation of reproduction. The relevance of changes in plasma testosterone to the changes in VFI in this model of the gonadal-intact ram are currently being investigated.

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