Hypothalamic control of photoperiod-induced cycles in food intake, body weight, and metabolic hormones in rams

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Received 12 June 2000; accepted in final form 12 February 2001

Lincoln, Gerald A., Stewart M. Rhind, Sueli Pompolo, and Iain J. Clarke. Hypothalamic control of photoperiod-induced cycles in food intake, body weight, and metabolic hormones in rams. Am J Physiol Regulatory Integrative Comp Physiol 281: R76–R90, 2001.—This study used a hypothalamic-pituitary disconnected (HPD) sheep model to investigate the central regulation of long-term cycles in voluntary food intake (VFI) and body weight (BW). VFI, BW, and circulating concentrations of metabolic hormones [α-melanocyte-stimulating hormone (α-MSH), insulin-like growth factor-1 (IGF-1), insulin, and leptin] were measured in HPD and control Soay rams exposed to alternating 16 wk periods of long and short days for 80 wk. In the controls, the physiology was cyclical with a 32-wk periodicity corresponding to the lighting regime. VFI and BW increased under long days to a maximum early into short days, and there were associated increases in blood concentrations of α-MSH, insulin, and leptin. In the HPD rams, there were no significant photoperiod-induced changes in any of the parameters. VFI increased after surgery for 8 wk and then gradually declined, although BW increased progressively and the HPD rams became obese. Concentrations of α-MSH, insulin, and leptin in peripheral blood were permanently increased (>200%), and levels of IGF-1 decreased (<55%). The HPD lesion effectively destroyed the entire median eminence [no nerve terminals immunostained for tyrosine hydroxylase (TH) and gonadotropin-releasing hormone] and the adjacent arcuate nucleus [no perikarya immunostained for proopiomelanocortin or TH, and no cells expressed neuropeptide Y mRNA]. The results support the conclusion that arcuate hypothalamic systems generate long-term rhythms in VFI, BW, and energy balance.

SEASONAL UNGULATES, IN COMMON with many other mammals adapted to cold and temperate climates, express conspicuous, long-term cycles in voluntary food intake (VFI) and body weight (BW), in addition to changes in reproduction, pelage, and other characteristics (34, 47). These cycles are robust in deer and feral breeds of sheep but are also evident in domesticated breeds of sheep, goats, and cattle, expressed as ancestral characteristics (15, 23). The generalized pattern is that VFI and BW increase from spring to autumn and decrease in winter with a specific seasonal pattern in males, females, and castrate males. Many experimental studies also indicate that the annual cycle in day length is used to dictate the timing of these cycles.

In ungulates housed indoors, transfer from short to long days provokes an increase in VFI, and exposure to alternating 3–6 monthly periods of long and short days “drives” the cycle in VFI and BW with a corresponding pattern (34, 38). In addition, there is evidence that long-term cycles in food intake, growth, and pituitary hormone secretion persist in sheep and deer living under constant conditions (10, 23, 32) and in animals rendered unresponsive to photoperiod by pinealectomy (44, 62). This supports the view that the seasonal VFI-BW cycle is generated endogenously as a circannual rhythm, a feature that has been experimentally investigated most thoroughly in long-lived, seasonal rodents (56, 60, 77). Thus under natural conditions, the response to the annual cycle in day length acts to entrain the period of the endogenous rhythm to exactly 12 mo and to set the phase to the time of year. The anabolic-growth phase is usually timed to summer when food is abundant, and the reciprocal catabolic-anorexia phase is timed to winter when food is scarce. These timing processes permit anticipation of the seasonal events in the environment and are clearly adaptive (51).

The neural mechanisms that control the expression of long-term cycles in VFI and BW are presumed to reside within the hypothalamus, particularly in the neural circuits of the medial, lateral, and paraventricular hypothalamus (3). Further, the effects of photoperiod are likely to be mediated through changes in the daily rhythm of melanatonin secretion from the pineal gland (2). These concepts are tentatively supported by experiments showing that lesions in the ventromedial and lateral hypothalamus disrupt the regulation of VFI and energy balance (3, 4, 5) and that lesions in the medial and rostral hypothalamus alter expression of...
long-term cycles in BW, prolactin secretion, and other seasonal characteristics (17, 55, 58, 59, 65, 68). Evidence that melatonin acts centrally is provided by the observation in sheep that placement of microimplants of melatonin in the mediobasal hypothalamus, but not in other sites in the hypothalamus and pituitary gland, induces a full spectrum of short-day responses including a phase advance of the BW cycle (39, 45).

Cells in the ventromedial and caudal hypothalamus express a low abundance of high-affinity melatonin receptors (12, 48) and may mediate the effects of photoperiod on the metabolic axis. In addition, recent studies have described seasonal or photoperiod-induced changes in the activity of genes encoding neuropeptides in the mediobasal hypothalamus, particularly neuropeptide Y (NPY) and proopiomelanocortin (POMC), which could dictate the appetite cycle (6, 13, 30, 71). Other carefully controlled studies in sheep and hamsters, however, have failed to demonstrate such an association (1, 63). Overall, the available data support the view that, although the hypothalamus is the site of integration of central and peripheral signals, multiple control pathways are likely to be involved in the generation and timing of the long-term cycles in VFI and BW.

The purpose of the current study was to utilize a hypothalamic-pituitary disconnected (HPD) sheep model to further investigate the central control of VFI and BW. In the standardized HPD surgery, the median eminence (ME) is visualized under an operating microscope and the neural tissue of the internal and external zones is extirpated. The pars tuberalis and associated vasculature are preserved to provide vital blood supply to the pars distalis, and a physical barrier is inserted separating the pituitary gland to the pars distalis, and a physical barrier is inserted to separate the pituitary gland and brain (14). Studies in Soay rams have shown that the HPD lesion permanently disrupts the expression of photoperiod-induced cycles in gonadotropin secretion and gonadal activity but spares the regulation of prolactin and the pelage axis (41). The prediction for the VFI-BW axis is that if melatonin acts on both central and peripheral tissues, and if multiple systems regulate the seasonal physiology and behavior, the HPD lesion would disrupt some but not all long-term cyclicity.

The first aim was to measure the long-term changes in VFI, BW, and blood concentrations of α-melanocyste-stimulating hormone (α-MSH), insulin, leptin, and insulin-like growth factor-1 (IGF-1) in HPD and control rams. This would show whether the lesion blocks the expression of both the photoperiod-induced and endogenously generated rhythmicity. Peripheral α-MSH was selected for study because it is the principal POMC peptide derived from the melanotropes of the pars intermedia and is implicated in the seasonal regulation of carbohydrate and fat metabolism (40). Insulin and IGF-1 were selected as metabolic hormones with multiple effects, and leptin was selected as a key product of adipocytes involved in the negative feedback regulation of food intake (33). As an adjunct to the study, the animals received insulin and glucose provocation tests during the increasing and decreasing phase of the VFI-BW cycle in controls, to assess possible changes in glucose homeostasis.

The second aim was to establish whether the HPD surgery destroyed both the ME and the adjacent arcuate nucleus (ARC) of the hypothalamus, a site particularly implicated in the regulation of VFI and energy balance (52). To this end, brain tissues were collected from all animals, and the extent of the HPD lesion within the mediobasal hypothalamus was investigated using immunocytochemistry and autoradiography for various neuroendocrine markers [NPY, POMC, gonadotropin-releasing hormone (GnRH), tyrosine hydroxylase (TH)].

**Materials and Methods**

**Animals.** The animals used in these experiments were adult rams of the Soay breed of feral sheep that express pronounced photoperiodically regulated cycles in VFI, BW, and other seasonal characteristics (34, 38). Two similar groups of eight animals (age: 27 mo; mean BW: 39.3 ± 1.0 kg SE at start of experiment) were housed permanently in adjacent light-controlled rooms. The animals were individually penned with visual and tactile contact between neighbors and received a constant daily ration (500 g) of dried grass nuts, with hay and water ad libitum. The animals were exposed to alternating 16 weekly periods of long days [16 h light (L): 8 h darkness (D); 16 L:8 D, LD] and short days (8 L:16 D, SD) to induce and entrain the long-term biological cycles before the start of the experiment. The light intensity was ~160 lux at the animals’ eye level, and the light adjustments were achieved by abruptly changing the time of lights out by 8 h; dim red light (<5 lux) was provided during the dark phase.

**Experimental manipulations.** Rams in the two adjacent rooms formed the two experimental groups. In group 1 (HPD rams, n = 8), all animals received a HPD operation midway through a period of LD. This coincided with the increasing phase of the BW cycle. In group 2 (control rams, n = 8), one-half of the animals received a sham HPD operation at the same time as the surgery in the HPD group (n = 4), and one-half of the animals received no operation (n = 4). The HPD operations were performed under a general anaesthetic by the method of Clarke et al. (14) as described previously (41). Briefly, this involved a left paramedial, transnasal, transsphenoidal approach to visualize the stalk of the pituitary gland using an operating microscope, entering the ME above the portal vasculature and evacuating the neural tissue of the tuber cinereum. A small sheet of aluminium foil was placed in the evacuated space to form a barrier between the hypothalamus and the pituitary gland, and to complete the 1- to 2-h operation the cavity in the sphenoid bone was blocked with gelatin sponge and sealed with dental acrylic. The sham HPD operations involved the same initial surgery to expose the pituitary stalk without entering the infundibulum. All rams recovered well after the operation. The HPD rams showed the expected clinical signs of the ablation of hypothalamic input to the pituitary gland including polyuria and gonadal regression but received no hormone replacement therapy.

The experimental observations extended over a period of 80 wk during which time the animals continued to be exposed to alternating 16 weekly periods of LD and SD. The period from week 16 to week 80 was designed to induce two complete 32-week cycles in physiology in the control rams (Fig. 1). The
animals remained in good body condition throughout, although one small HPD animal died after 72 wk due to liver and kidney failure. The results for this animal were included up to 1 mo before death before the overt illness developed. One control animal developed septicemia shortly after the study and was destroyed; thus material from only 7 rams/group was available at final autopsy.

Routine monitoring. To measure the long-term changes in peripheral hormone concentrations, a blood sample was collected from each animal by vacutainer from the jugular vein twice weekly. The blood samples were heparinized, and the plasma was separated by centrifugation. Three aliquots of each sample were stored at -20°C, and these were used to measure the hormonal concentrations by RIA at the end of the study. The multiple aliquots were used to minimize the number of times any plasma sample was thawed and refrozen for different assays.

Every week VFI was measured over a period of 24 h by providing a known weight of dried grass pellets ad libitum, without hay, and subtracting the weight of food remaining at the same time the following day. The food hoppers were designed to minimize food wastage. Every 4 wk the animals were weighed using a large animal cage balance.

Provocation tests. To assess glucose homeostasis, HPD and control rams received standardized insulin and glucose provocation tests under both SD (experimental week 60, during the declining phase of the BW cycle) and LD (experimental week 76, during the increasing phase of the BW cycle). These tests were conducted during the early light phase, and the introduction of the daily food ration was delayed until after the tests. The provocation tests involved the administration of a bolus injection of insulin (4.0 IU/ram iv) given on one selected day and a bolus injection of glucose (8.0 g/ram iv) given 3 days later. This was repeated under each of the two photoperiods. Blood samples were collected at -20, -10, 0, 10, 20, 30, 40, 51, 60, 75, 90, 105, and 120 min relative to the time of the injections. The doses of insulin and glucose selected were based on previous studies in sheep and cattle (61, 69). To permit the collection of the serial blood samples, each animal was fitted with an indwelling cannula inserted into the jugular vein before the study and kept patent with heparinized saline (10,000 IU sodium heparin/l 0.9% NaCl). The blood samples were placed in heparinized tubes, and the plasma was separated by centrifugation and stored at -20°C until the concentrations of insulin and glucose were measured.

RIAs. For the routine blood samples, the concentrations of α-MSH were measured by RIA (40). The assay validated for sheep plasma used the antibody R6PB raised in rabbits against synthetic α-MSH. The antibody showed >75% cross-reactivity with des-acetylated α-MSH, 0.15% cross-reactivity with 1–39 human ACTH, and no detectable cross-reaction with β-MSH, human gamma lipotropin, and β-endorphin (β-END; Ref. 25). The lower limit of detection (10% decrease in binding relative to binding with no addition of the standard α-MSH) was 25 pM/l plasma, and the intra- and inter-assay coefficients of variation (CV) were 14 and 16%, respectively, based on quality control samples run in 20 assays.

The concentrations of IGF-1 and insulin were also measured using RIA (11, 48). For the IGF-1 assay, the lower limit of detection was 35 ug/l plasma, and the intra- and interassay CV were 8.8 and 12%, respectively; the corresponding values for insulin assays were 2.6 mU/l and 9.1 and 15%, respectively.

Leptin concentrations were measured in a single weekly plasma sample from each animal using the multispecies leptin assay kit (Linco Research; Biogenesis, Poole, Dorset, UK). This kit produced the predicted results for the quality controls (+10%) and was validated for use with the sheep plasma based on a fourfold serial dilution of plasma collected from obese HPD rams (predicted high concentrations), which produced values closely parallel with the leptin standard curve. The addition of the standard preparation of leptin to plasma from hypophysectomized sheep, to produce concentrations in the physiological range (0–20 ng/ml), gave values within 8.8 ± 1.3% of those predicted, using the assay.

The blood plasma samples collected during the insulin and glucose provocation tests were assayed for insulin (as above) and for glucose, using a continuous flow glucose oxidase method (64) with a limit of detection of 0.51 mM/l and intra- and interassay CV of 2.6 and 6.3% respectively.

Autopsy studies. At the end of the study the animals were killed at 8 wk into a period of LD, using an overdose of a barbiturate (Euthatal, Rhone Merieux, Essex, UK). Heparin (25,000 IU) was administered systemically immediately before death and for each animal the entire head was perfused via the two carotid arteries with heparinized 0.9% saline (25,000 IU/l) using a gravity-feed system to remove blood (~1.5 l saline/10 min). Each head was then fixed using 4% paraformaldehyde in 0.1% phosphate buffer (2.0 l/15 min), followed by 20% sucrose in the same fixative (1.0 l/10 min). After fixation, the brain and pituitary gland were removed from the skull. A central block of brain tissue including the entire hypothalamus (~5 × 2.5 × 2.0 cm) was equilibrated in 30% sucrose in 4% paraformaldehyde fixative (100 ml). After 3–5 days at 4°C, the tissues were frozen at ~70°C until used

![Diagram](http://ajpregu.physiology.org/Downloadedfromhttp://ajpregu.physiology.org/)}
for histology. For all animals, the carcass was fully dissected and the weights of the body organs and main fat deposits were recorded.

**Histology.** The hypothalamic blocks from all animals were sectioned (40 μm) in coronal orientation on a cryostat, and all sections between the rostral margin of the optic chiasm and the mammillary bodies were stored in sequence in 2% paraformaldehyde-cryoprotectant solution in coded 94-well plates at –20°C. Selected sections from the seven control and HPD rams were mounted onto coated slides and stained with cresyl violet for camera lucida drawings. These were used to define macroscopically the extent of surgical removal of tissue from the mediobasal hypothalamic region in HPD animals.

**Immunohistochemistry.** The degree to which the HPD lesions destroyed or compromised the function of the ME and ARC was investigated by immunocytochemistry for three markers, namely adrenocorticotropic (ACTH; as an index of the presence of POMC neurons), GnRH, and TH. The specific ACTH antiserum was a gift from Dr. Ben Canny (Dept. of Physiology, Monash University), and the specific GnRH antiserum (LR-2) was kindly provided by Dr. R. Benoit (University of Montreal). The TH antiserum was obtained from a commercial source (Boeringer-Mannheim, Germany). All antisera were raised in rabbits. Free-floating 40-μm sections were mounted onto coated slides and allowed to dry overnight. Sections were then immersed in 0.01 M citrate buffer, pH 6.0, and given two microwave treatments each of a 5-min duration. Sections were then left in solution for 20 min before receiving four 5-min washes in 0.05 M phosphate buffered saline. All subsequent incubations were carried out at room temperature unless otherwise stated. To eliminate endogenous peroxidase activity, sections were incubated in 3% hydrogen peroxide plus 0.1% sodium azide (20 min), followed by rinses in PBS plus 0.3% Triton X-100 and 1/500 streptavidin to block endogenous biotin (60 min). Primary antibody incubations were carried out using diluted antisera (ACTH antibody, 1/5,000; GnRH antibody, 1/10,000; TH antibody, 1/500) for 48 h at 4°C with agitation, followed by an incubation of 8 h at room temperature. Secondary antibody incubation was carried out after four 5-min washes, using biotinylated goat anti-rabbit antibody 1/40 (Vector, Burlingame, CA) for 24 h. Sections were then incubated with streptavidin biotinylated horse radish peroxidase complex 1/600 (Amersham, Bucks, UK) for 1 h. Peroxidase was visualized using 0.05% 3,3 diaminobenzidine with 0.003% hydrogen peroxide as substrate.

**In situ hybridization.** To further illustrate the extent of the HPD lesion, the expression of NPY mRNA was investigated by in situ hybridization. Selected sections were removed from cryoprotectant solution, mounted onto Super Frost Plus slides (Menzel-Glaser, Germany), and dried at room temperature overnight. In situ hybridization was performed using a 511-bp rat NPY insert (29) in pBSM13 labeled with 35S-dUTP (NEN Life Science Products, Boston, MA) following the method of Simmons et al. (70). The amplification, purification, and linearization of plasmid DNA were performed using standard techniques (67). Hybridization was carried out in a humid chamber at 53°C for a minimum of 16 h. After the posthybridization treatment, slides were dipped in Ilford K5 photographic emulsion (Ilford Australia, Mount Waverly, Australia) and exposed at 4°C for 14 days. The exposed film was developed using Ilford Phenisol X-ray developer and stop bath Hypam fixer.

**Statistical analysis.** In the control rams, there were no observed differences between the sham-operated and nonoperated rams in any of the recorded parameters and the data for the two groups were combined. Long-term changes in the plasma hormone concentrations in control and HPD rams for the different phases of the experiment were compared using two-way ANOVA with repeated measures. To summarize the changes with respect to photoperiod, group mean values (± SE) were calculated for four phases (phase 1: SD 0–8 wk; phase 2: SD 8.5–16 wk; phase 3: LD 0–8 wk; phase 4: LD 8.5–16 wk). The selection of the time windows was based on time lags involved in the response to changes in photoperiod and the development of photorefractoriness (38). The mean values were determined for each individual for the first photoperiod-induced cycle (experimental weeks 16–48) and the second cycle (experimental weeks 49–80), and these were used to calculate an overall mean and then the group means ± SE (Table 1). The latency of response to the insulin and glucose provocation tests was defined as the time from

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**Table 1. VFI and blood plasma endocrine parameters in control and HPD Soay rams at 4 phases of an artificial lighting regimen of alternating 16 weekly periods of short days and long days**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Phase 1 (0–8 wk)</th>
<th>Phase 2 (9–16 wk)</th>
<th>Phase 1 (0–8 wk)</th>
<th>Phase 2 (9–16 wk)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VFI, kg/day</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>1.61 ± 0.15a</td>
<td>0.58 ± 0.09b</td>
<td>0.62 ± 0.08b</td>
<td>1.27 ± 0.10a</td>
</tr>
<tr>
<td>HPD</td>
<td>1.24 ± 0.09</td>
<td>1.21 ± 0.09‡</td>
<td>1.12 ± 0.08‡</td>
<td>1.05 ± 0.10</td>
</tr>
<tr>
<td>α-MSH, pmol/l plasma</td>
<td>127.58 ± 26.08a</td>
<td>51.53 ± 10.68b</td>
<td>22.60 ± 2.86c</td>
<td>27.39 ± 3.97c</td>
</tr>
<tr>
<td>C</td>
<td>149.30 ± 29.07</td>
<td>129.05 ± 29.66†</td>
<td>113.22 ± 27.35‡</td>
<td>103.10 ± 25.00‡</td>
</tr>
<tr>
<td>IGF-I, μg/l plasma</td>
<td>935.65 ± 35.69</td>
<td>917.01 ± 40.96</td>
<td>992.06 ± 53.24</td>
<td>865.36 ± 29.21</td>
</tr>
<tr>
<td>C</td>
<td>514.61 ± 34.79‡</td>
<td>457.69 ± 34.40</td>
<td>496.05 ± 38.43</td>
<td>549.86 ± 45.06</td>
</tr>
<tr>
<td>Insulin, mIU/l plasma</td>
<td>49.54 ± 6.54a</td>
<td>40.01 ± 4.68b</td>
<td>34.68 ± 3.84b</td>
<td>43.36 ± 4.98a</td>
</tr>
<tr>
<td>C</td>
<td>86.44 ± 13.54‡</td>
<td>89.64 ± 16.25‡</td>
<td>95.45 ± 17.05‡</td>
<td>98.57 ± 17.71‡</td>
</tr>
<tr>
<td>Leptin, μg/l plasma</td>
<td>2.17 ± 0.10a</td>
<td>1.98 ± 0.07b</td>
<td>1.82 ± 0.04b</td>
<td>2.28 ± 0.11a</td>
</tr>
<tr>
<td>C</td>
<td>4.62 ± 0.40</td>
<td>5.26 ± 0.46</td>
<td>5.07 ± 0.48</td>
<td>5.89 ± 0.58</td>
</tr>
</tbody>
</table>

Values are means ± SE. VFI, voluntary food intake; C, control; HPD, hypothalamo-pituitary disconnected; short days, 8 h light; 16 h dark; long days, 16 h light: 8 h dark; α-MSH, α-melanocyte-stimulating hormone; IGF-I, insulin-like growth factor-1. Light-cycle phase comparison: values denoted by different superscript letter are significantly different (P < 0.05, ANOVA). Group comparison: values denoted by asterisks are significantly different (*P < 0.05, †P < 0.01, ‡P < 0.001, ANOVA).
the test injection to the maximum or minimum concentration of insulin or glucose in the blood plasma. The magnitude of the response was defined as the mean blood plasma concentration of each parameter for the total 2-h period after the injection, minus the mean pretreatment value (i.e., area under the response curve). In all cases this was determined for each individual, and the significance of differences between photoperiod and treatments (control compared with HPD) was assessed by two-way ANOVA. The correlation coefficients were calculated using Cricket Graph software (Cricket Graph, Philadelphia, PA).

RESULTS

Extent of the HPD lesion. The macroscopic appearance of sections through the hypothalamus of one control and one HPD ram, based on camera lucida drawings, is illustrated in Fig. 1. The HPD surgery effectively removed the internal and external zones of the entire ME and adjacent tissue in the mediobasal hypothalamus in all animals. The lesion extended on the midline from the caudal margin of the optic chiasma to close to the mammillary bodies. It removed a proportion of the region of the ARC without macroscopically affecting the lateral, dorsal, and paraventricular hypothalamus. The base of the third ventricle was variably expanded such that cerebral spinal fluid would have been in direct contact with the foil barrier placed between the hypothalamus and pituitary gland.

Immunostaining for GnRH and TH was used to characterize the tissues of the ME in the two groups of animals (Fig. 2). In all control rams, staining for GnRH was most dense in the lateral external (neurosecretory) zone of the ME, and the vesicular appearance of the axons in the terminal fields in the external zone was clearly evident (Fig. 2, A and B). TH immunostaining was also evident throughout the internal and external ME indicative of extensive catecholamine innervation (Fig. 2, E and F). In the HPD rams, there were only small remnants of tissue in the region of the ME. This tissue had no defined structure in any of the HPD rams and was not immunoreactive for GnRH and TH (Fig. 2, C, D, E, and H). The extent of damage to the ARC adjacent to the ME was investigated using immunostaining for ACTH (index of POMC neurones) and TH and radiolabeling by in situ hybridization for NPY (Fig. 3). In control rams, POMC cells were abundant throughout the ARC (Fig. 3A). TH cells were localized to the lateral border (Fig. 3C), and NPY cell bodies were concentrated to the wall of the third ventricle (Fig. 3, E and G). In the HPD rams, there was no immunostaining for POMC and TH in the region of the ARC (Fig. 3, B and D), and only the occasional NPY cells were detected by in situ hybridization (Fig. 3, F and H).

To assess whether there was a relationship between the apparent extent of the lesion, the HPD rams were ranked according to the degree of destruction and/or distortion of the normal architecture of the mediobasal hypothalamus. This was based on the macroscopic appearance of the tissues for each animal and the evidence of remnants of the ARC characterized by the presence of NPY cells. This rank order was not significantly correlated with the individual variation in BW or weight of kidney and omental fat in the HPD rams at autopsy (see below).

VFI, live BW, and blood α-MSH. The long-term changes in VFI, BW, and peripheral plasma concentrations of α-MSH for the control and HPD rams throughout the 80-wk experiment are illustrated in Fig. 4. In the control rams there were marked cyclical changes in all three parameters. VFI increased under LD, remained elevated during the first 8 wk under SD, and then declined under SD. A similar pattern was repeated in the second cycle, with a parallel change in BW. The interval from the first maximum to the second maximum was 31.8 ± 0.5 and 32.0 ± 0.0 wk for VFI and BW, respectively, corresponding closely to the period of the driving light regimen.

Plasma concentrations of α-MSH were basal under LD, were increased late under LD, and were maximal during the first half of the exposure to SD (>800% basal). The highest values coincided with the peak in the BW cycle and then declined later in SD in parallel with the decline in VFI and BW (Fig. 4). The interval from the maximum in the first cycle to the maximum in the second cycle was again very close to 32 wk, and the changes with respect to the photoperiod regimen were highly significant (Table 1).

In the HPD rams, cyclical changes in VFI, BW, and blood concentrations of α-MSH were not apparent. VFI remained similar to control values for the first 16 wk after the HPD operation and then gradually declined throughout the experiment to a mean value midway between the hyper- and hypophagic states of the controls. The plasma concentrations of α-MSH increased immediately after the HPD surgery to a maximum at ~8 wk and then gradually declined. The concentrations remained markedly elevated relative to controls (mean, 216% of controls) and with a long-term pattern similar to that for VFI (Fig. 4). Conversely, BW increased progressively in the HPD rams. The animals were permanently heavier (P < 0.05) than the controls from 16 wk after the HPD surgery and were notably obese by the end of the study. In the HPD animals, there were no significant changes in VFI or α-MSH associated with the shifts in photoperiod (Table 1).

Post mortem, the greater BW of HPD than control rams (127% of control) was found to be attributable to greater kidney and omental fat stores (168% of control), heavier skin and fleece (149% of control), and a marginally heavier liver (129% of control) (Table 2).

Plasma IGF-1, insulin, and leptin. The long-term changes in the plasma concentrations of IGF-1, insulin, and leptin are illustrated in Fig. 5 and summarized in Table 1. In the control rams, the plasma concentrations of IGF-1 were relatively stable and exhibited no consistent cyclicity in relation to photoperiod. Mean plasma concentrations of insulin and leptin were higher during the first 8 wk under SD (SD, phase 1) compared with the corresponding period under LD (LD, phase 1, Table 1). The cycles in plasma insulin and leptin concentration roughly paralleled the cycles
in BW. Within the control group, overall mean plasma concentrations of insulin and leptin were positively correlated with mean BW (log plasma insulin against BW, $r^2 = 0.66$, not significant; log plasma leptin against BW, $r^2 = 0.81$, $P < 0.01$).

In the HPD rams, there were no consistent changes in the plasma concentrations of IGF-1, insulin, and leptin associated with photoperiod (Fig. 5; Table 1). Plasma concentrations of IGF-1 were immediately lower than the controls from the time of the HPD surgery (54% of control, Table 1). Plasma concentrations of insulin and leptin, in contrast, were not significantly increased until after 16 wk (220–250% of control, Table 1). Mean live BWs of the two groups also diverged at this time (Figs. 4 and 5). Within the HPD group, overall mean plasma concentrations of insulin were positively correlated with mean live BW (log insulin against BW, $r^2 = 0.760$, $P < 0.05$), and plasma concentrations of insulin were positively correlated with mean plasma leptin (log insulin against log insulin, $r^2 = 0.735$, $P < 0.05$).

**Insulin and glucose provocation tests.** The effects of a bolus injection of insulin (4.0 IU/ram iv) on the plasma concentrations of insulin and glucose in the control and HPD rams are summarized in Fig. 6. In the control rams, the injection of insulin induced an immediate decline in plasma glucose that was most pronounced at 30 min, with recovery to normal values by 120 min.
The response pattern was essentially identical under LD and SD (Fig. 6). In the HPD rams, the same dose of insulin also induced hypoglycemia under both photo-periods. The latency to the minimum glucose concentration was similar in the HPD rams compared with controls, but the recovery was consistently delayed (Fig. 6). This was reflected in a significantly \( (P < 0.001) \) larger total decline in glucose concentrations (i.e., area under the response curve) in the HPD rams.

The effects of the bolus injections of glucose (8.0 g/ram iv) on the plasma concentrations of insulin in the control and HPD rams are summarized in Fig. 7. In the controls, the glucose induced an immediate increase in plasma insulin levels that were maximal after 10–20 min and returned to normal values by 120 min. The response was similar under both LD and SD and was also similar in the HPD rams (Fig. 7).

**DISCUSSION**

The study clearly demonstrates that HPD surgery, and the associated destruction of the ME and ARC, disrupted long-term rhythmicity in this highly seasonal breed of sheep. The HPD rams showed no cycles in VFI, BW, and blood concentrations of \( \alpha \)-MSH, insulin, and leptin associated with the switches in photo-period, or no conspicuous longer term rhythms in individuals that might represent the persistence of endogenously generated circannual rhythms. This was in marked contrast to the controls in which the seasonal...
cycles were robust and precisely entrained by the artificial lighting regimen to a 32-wk periodicity. In the HPD group, the disruption affected behavior (VFI), pituitary function (α-MSH), peripheral metabolic signals (insulin and leptin), and whole body responses (BW). This supports the view that long-term rhythms are generated centrally and expression depends on a functionally intact hypothalamo-pituitary system.

Previous studies have demonstrated that HPD rams continue to express normal patterns in prolactin secretion in response to switches in photoperiod (41). This contrasts with the effect on the metabolic axis described here; thus it is unlikely that prolactin plays a key role in the generation of the long-term cycles in VFI and BW (37, 66), unless the response to prolactin also requires a functionally intact hypothalamo-pituitary system.

Table 2. LBW and organ weights in control and HPD Soay rams killed at 8 weeks into long days

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control Weight</th>
<th>%LBW</th>
<th>HPD Weight</th>
<th>%LBW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Live body, kg</td>
<td>39.64 ± 1.92</td>
<td>—</td>
<td>50.25 ± 1.54†</td>
<td>45.76 ± 1.13</td>
</tr>
<tr>
<td>Carcass, kg</td>
<td>18.59 ± 0.74</td>
<td>47.01 ± 0.58</td>
<td>23.12 ± 1.26*</td>
<td>9.87 ± 0.34*</td>
</tr>
<tr>
<td>Skin and fleece, kg</td>
<td>3.31 ± 0.29</td>
<td>8.34 ± 0.45</td>
<td>4.93 ± 0.12†</td>
<td>3.79 ± 0.36†</td>
</tr>
<tr>
<td>Kidney fat, kg</td>
<td>1.11 ± 0.11</td>
<td>2.79 ± 0.20</td>
<td>1.88 ± 0.15†</td>
<td>6.24 ± 0.34†</td>
</tr>
<tr>
<td>Omentum fat, kg</td>
<td>1.87 ± 0.16</td>
<td>4.67 ± 0.20</td>
<td>3.13 ± 0.18†</td>
<td>3.79 ± 0.36†</td>
</tr>
<tr>
<td>Rumen and intestines, kg</td>
<td>6.53 ± 0.35</td>
<td>16.54 ± 0.72</td>
<td>8.39 ± 0.62*</td>
<td>16.74 ± 1.31</td>
</tr>
<tr>
<td>Liver, g</td>
<td>554.3 ± 43.4</td>
<td>1.397 ± 0.080</td>
<td>716.7 ± 58.1*</td>
<td>1.415 ± 0.085</td>
</tr>
<tr>
<td>Lungs, g</td>
<td>388.6 ± 52.4</td>
<td>0.964 ± 0.086</td>
<td>413.3 ± 13.6</td>
<td>0.823 ± 0.013</td>
</tr>
<tr>
<td>Heart, g</td>
<td>179.9 ± 8.8</td>
<td>0.454 ± 0.008</td>
<td>164.3 ± 6.35</td>
<td>0.327 ± 0.006†</td>
</tr>
<tr>
<td>Kidney-combined, g</td>
<td>132.3 ± 5.1</td>
<td>0.340 ± 0.025</td>
<td>149.1 ± 14.6</td>
<td>0.296 ± 0.026</td>
</tr>
<tr>
<td>Pancreas, g</td>
<td>46.9 ± 4.7</td>
<td>0.120 ± 0.014</td>
<td>48.0 ± 5.3</td>
<td>0.097 ± 0.011</td>
</tr>
<tr>
<td>Spleen, g</td>
<td>397.1 ± 81.1</td>
<td>1.037 ± 0.219</td>
<td>520.0 ± 43.4</td>
<td>1.033 ± 0.072</td>
</tr>
<tr>
<td>Thyroid-combined, g</td>
<td>3.06 ± 0.19</td>
<td>0.0078 ± 0.0005</td>
<td>2.41 ± 0.35</td>
<td>0.0047 ± 0.0003†</td>
</tr>
<tr>
<td>Adrenal-combined, g</td>
<td>2.93 ± 0.07</td>
<td>0.0074 ± 0.0003</td>
<td>3.31 ± 0.23</td>
<td>0.0065 ± 0.0001</td>
</tr>
<tr>
<td>Pituitary, g</td>
<td>0.652 ± 0.033</td>
<td>0.0016 ± 0.0001</td>
<td>0.395 ± 0.039†</td>
<td>0.0008 ± 0.0001†</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 7/group. LBW, live body weight. Group comparison: *P < 0.05, †P < 0.001, ANOVA.
itary system. The observation that the HPD lesion blocks the photoperiodic control of BW, but spares the control of prolactin, indicates that the melatonin signal that relays the effects of photoperiod most probably acts at different sites to control these responses. The mediobasal hypothalamus is the most likely site of melatonin regulation of cycles in VFI and BW (45), and the pars tuberalis with its high expression of melatonin receptors is the most likely site of melatonin regulation of cycles in prolactin secretion (42).

The histological analysis of the extent of the HPD lesion is important in interpreting these results. The HPD animals were killed more than 2 yr after the surgery. At autopsy it was found that the smaller pituitary gland was physically separated from the hypothalamus with minimal interconnecting tissue. The neurosecretory component of the ME was totally destroyed in all animals as indicated by the lack of immunostaining for GnRH and TH in the tissue rudiments. Neural cells bodies expressing NPY, POMC, and TH were notably absent or very scarce in the adjacent ARC region of the basal hypothalamus.

The lesion therefore destroyed the function of the ARC, as well as disconnecting the pituitary stalk. The loss of the neurosecretory zone of the ME is consistent with the permanent regression of the testes that occurred in the HPD rams. This would be due to the loss of GnRH secretion from the ME that is essential for the stimulation of gonadotropin secretion (14). It is also consistent with the observed hypersecretion of α-MSH in the HPD rams. α-MSH in the peripheral blood is derived primarily from the melanotropes of the pars intermedia of the pituitary gland, and enhanced secretion in HPD animals is thought to result from the loss of a dopaminergic inhibitory regulation by the hypothalamus (50, 72). The failure of HPD rams to respond to photoperiod for the VFI-BW axis may have resulted from damage to the caudal ARC (premammillary hypothalamus) where cells express melatonin receptors and appear to mediate the central effect of photoperiod on seasonal responses (48). Overall, the results support the view that an intact ME-ARC is essential for the generation of long-term rhythms in VFI and BW.
The development of obesity was the other notable characteristic of the HPD rams. At the end of the study, the HPD animals were ~25% heavier than the controls. This was due to an increase in kidney and omental fat stores and a heavier skin and fleece. The BW of the HPD rams diverged from the controls 16–24 wk after the surgery when the HPD group failed to become hypophagic in response to the change from long to short days. BW in the HPD rams increased progressively throughout the study, whereas VFI gradually declined to values midway between the cyclic extremes of the controls. The development of obesity without overeating clearly indicates that the HPD surgery interfered with the regulation of energy homeostasis resulting in a permanent positive energy balance.

Several different mechanisms can be invoked to explain this change in energy balance. The most obvious is the loss of the drive to the reproductive axis and the associated reduction in energy expenditure. The testes in HPD animals regress to a size markedly smaller than in intact controls, even at the nadir of their sexual cycle, and circulating concentrations of testosterone are permanently very low (43). The HPD animals are thus similar to castrates in terms of gonadal steroid secretion. Castration in ungulates is well-known to affect body composition with a decrease in lean muscle mass, an increase in fat, and a change in the distribution and chemical composition of fat (19, 24, 57). These changes may be attributed to the withdrawal of the anabolic effects of androgens and the reduction of energy expenditure due to a decline in sexual and aggressive behavior (53). These “castration” effects clearly contribute to the HPD phenotype; however, HPD rams also differ from castrates. Long-term castrated Soay rams housed indoors under similar conditions to the current animals generally have a lower mean BW than intact controls at the peak of the BW cycle and are not overtly rotund and obese like HPD rams (Lincoln, unpublished observations). Castrates also continue to express normally timed photoperiod-induced cycles in

![Graphs showing acute changes in blood plasma concentrations of glucose and insulin after a bolus injection of insulin](http://ajpregu.physiology.org/)

**Fig. 6.** Acute changes in blood plasma concentrations of glucose (A) and insulin (B) after a bolus injection of insulin (4.0 IU iv) in control (●) and HPD (○) Soay rams treated under long days (16 L:8 D; LD, left) and short days (8 L:16 D; SD, right). Data are means ± SE; n = 8.
VFI and BW, albeit at reduced amplitude compared with controls (34, 40).

Changes in the hypothalamic control of the pituitary gland, unrelated to the reproductive axis, most probably also contributed to the noncyclic, obese syndrome in the HPD rams. This includes alterations in the secretion of α-MSH from the pars intermedia. Peripherally circulating α-MSH is thought to play an important role in the peripheral regulation of carbohydrate and fat metabolism promoting seasonal lipogenesis. This is based on the close positive correlation between the seasonal activation of the pars intermedia and the BW cycle in the hypothalamic-intact Soay sheep (40). This association was also evident in the current study. α-MSH may affect lipogenesis via a direct action on adipocytes or via an indirect action in the pancreas to affect insulin secretion. In rodents, these tissues express melanocortin receptors (MC2 and MC5 receptors in adipocytes and MC4 receptors in pancreas; Refs. 9, 54). α-MSH is also strongly implicated in the central control of food intake acting through MC4 and MC5 receptors (16, 27), but it is not clear to what extent peripherally derived α-MSH would gain access into the brain to affect this system. Peripheral concentrations of α-MSH were positively correlated with the level of VFI in our sheep. Other pituitary POMC products may also promote lipogenesis. β-END is cosecreted with α-MSH and is increased in peripheral blood in various obese syndromes (20, 40), and β-cell tropin is a potent stimulator of insulin secretion (7). In the HPD rams, the pars intermedia becomes hypertrophied (50) and secretes increased amounts of POMC peptides; these are likely to be an important hormonal stimulus promoting obesity.

The disruption in growth hormone (GH) secretion also needs to be considered. In the normal intact animal, the synthesis and release of GH is regulated by the opposing effects of GH-releasing hormone and so-
matostatin released from the ME (74). The HPD lesion destroys these control systems (22). This is indicated by the absence of GH pulses in peripheral blood in HPD rams (46). GH normally acts in the liver to stimulate the production of IGF-1 and thereby promotes somatic growth (18). An immediate and persistent decrease in circulating IGF-1 concentrations was apparent in the HPD rams in the current study, presumably reflecting the decline in GH secretion. The HPD animals were already close to their full adult body size before the surgical ablation, and thus there was no opportunity to observe an overt blockade of somatic growth. The administration of physiological concentrations of IGF-1 has been shown to inhibit insulin secretion (36); thus the decline in IGF-1 release in the HPD rams may have contributed to the chronic increase in insulin secretion observed in these animals. Conversely, insulin affects the production of IGF-1 and IGF binding proteins by the liver (73), which may indicate that some components of peripheral homeostasis could persist in HPD animals. In the long term, the withdrawal of GH secretion is likely to result in a shift away from expenditure of energy in growth and repair and toward the storage of energy as fat.

The destruction of the ARC in the HPD rams is the final factor to be considered in the etiology of the HPD obese syndrome. The ARC forms a neural network with the other hypothalamic areas involved in the regulation of appetite. It is believed to play a central role in the control of appetite and feeding behavior through the synthesis of peptides including NPY, POMC peptide, cocaine- and amphetamine-regulated transcript, and the agouti-related peptide that potentially antagonizes the central effects of α-MSH (33). NPY has been shown to be a potent stimulator of food intake in sheep (52), although α-MSH is an inhibitor of food intake, at least in rodents (16, 27). NPY cells in the ARC express leptin receptors (75) and are thought to play an important role in the negative feedback control of appetite by responding to leptin derived from adipocytes, an index of fatness (8, 28). The ARC is thus an integrative center, and its removal would be expected to have various sequelae (26). With both stimulatory and inhibitory influences on appetite, it was impossible to predict how ablation of the ARC would affect feeding behavior. In the current study, the HPD lesion had a minimal effect on the general level of VFI, except for the acute aphagic-response immediately following surgery. The level of VFI was never outside the range expressed by the controls, supporting the view that a functional ARC is not required for the basic motivation of feeding behavior. The HPD rams were clearly abnormal, nonetheless, as they failed to adjust the VFI in the face of increasing obesity. The circulating concentrations of leptin were very high as expected in obese animals, but the signal failed to suppress appetite presumably due to destruction of the feedback system in the ARC.

With the development of chronic obesity in the HPD model, one expectation was that HPD rams would become insulin resistant. This seemed probable because all tissues would be exposed chronically to abnormal levels of insulin and glucose, thus affecting the responsiveness of the pancreas to glucose and the responsiveness of peripheral tissues to insulin. The results of the insulin and glucose provocation tests, however, failed to reveal differences between the HPD and control rams in either the acute uptake of glucose in response to insulin or the release of insulin in response to a bolus of glucose. This was despite clear evidence of the development of hyperinsulinemia in the HPD rams extending over more than a year. The only abnormality detected in the HPD rams was a consistent delay in the recovery to an euglycemic state after treatment with insulin. This may be explained by the inability of HPD animals to release glucocorticoids as a stress response to hypoglycemia (21); the release of glucocorticoids would be expected to promote glucogenesis to restore the euglycemia.

In conclusion, this integrative study clearly demonstrates that a standardized HPD lesion used experimentally in sheep totally disrupts the expression of photoperiod-induced rhythms in VFI, BW, and various associated hormones. The HPD lesion destroys the ME and the adjacent ARC but leaves the rest of the brain intact. The consequent changes in physiology and behavior result from 1) loss of the normal hypothalamic regulation of the pituitary gland and 2) loss of the ARC per se. The loss of the hypothalamic drive is likely to affect peripheral energy metabolism through changes in the release of hormones from the pars intermedia (e.g., α-MSH) and from the pars distalis (e.g., GH, gonadotropins). The loss of the ARC is likely to have central effects by altering feeding behavior and responsiveness to feedback signals from the periphery. The consequence is a failure to control the balance between food intake and energy expenditure and thus the development of a hyperinsulinemic, hyperleptinemic obese syndrome. Overall, the results support the conclusion that the ME-ARC is the final common pathway by which the multiple systems of the hypothalamus control energy balance.

**Perspectives**

Mutations in genes that affect the expression and/or function of POMC-derived peptides and their cognate receptors appear to be the major cause of familial, early-onset, chronic obesity in man (31, 35, 76). This highlights the importance of POMC-melanocortin signaling in normal regulation in the energy homeostasis. Studies in laboratory rodents indicate that POMC neurones in the ARC of the hypothalamus are focally involved. These secrete α-MSH, β-END, and other POMC peptides within the hypothalamus regulating feeding behavior, and these neurones are modulated by feedback signals from metabolism and fat stores as part of a homeostatic mechanism (54). The seasonal sheep model provides a different perspective. In this model, the concentrations of α-MSH in peripheral blood increase 20-fold from spring to autumn (40) or in response to short days (current study), a response that
is dependant on a functionally intact hypothalamus. The peripheral α-MSH is secreted largely from the melanotropes of the pars intermedia in sheep, and the cycle in α-MSH correlates closely with the long-term cycle in BW but not with other overt seasonal characteristics. Thus it is likely that pruititary α-MSH (or a related POMC peptide) plays a key role in the peripheral regulation of energy metabolism, promoting seasonal fattening in autumn. Melanocortin receptors are expressed in adipose tissue, pancreas, and other peripheral tissues involved in the control of energy metabolism; thus circulating α-MSH may act in a variety of tissues to promote seasonal lipogenesis. The observation that both central (ARC-based) and peripheral (pituitary-based) POMC-melanocortin signaling systems are likely to be critical to the control of long-term energy homeostasis in mammals. Mutations of POMC or melanocortin receptor genes thus interfere with both the central regulation of appetite, and the peripheral regulation of energy metabolism. The resulting, unregulated, permanent, positive energy balance, as in our HPD sheep, causes chronic obesity.

We are grateful to Norah Anderson, Joan Dockerty, Marjorie Thomson, and the staff at the Marshall Building for the collecting of blood samples and the long-term care of the animals; to Ian Swanston, Vivian Grant, Alda Pereira, and Mike Miller for expert technical assistance with the RIAs and the immunocytochemistry; and to Tom McPeters and Ted Pinner for the art work. Dr. Ben Canny (Department of Physiology, Monash University) generously provided the ACTH antiserum, and Dr. R. Benoit (University of Montreal) kindly donated the GnRH antiserum for the immunocytochemistry. Sten, Vivian Grant, Irene Cooper, Alda Pereira, and Mike Miller for blood samples and the long-term care of the animals; to Ian Swanston, Vivian Grant, Alda Pereira, and Mike Miller for expert technical assistance with the RIAs and the immunocytochemistry; and to Tom McPeters and Ted Pinner for the art work. Dr. Ben Canny (Department of Physiology, Monash University) generously provided the ACTH antiserum, and Dr. R. Benoit (University of Montreal) kindly donated the GnRH antiserum for the immunocytochemistry. Insulin and insulin antiserum for the RIA were provided by the National Hormone and Pituitary Programme, the National Institute of Diabetes and Digestive and Kidney Diseases, the National Institute of Child Health and Human Development, and the U.S. Department of Agriculture.

REFERENCES


