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Blood-Brain Leptin Transport and Appetite and Reproductive Neuroendocrine Responses to Intracerebroventricular Leptin Injection in Sheep: Influence of Photoperiod

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Impaired anorectic actions of leptin may be due to intrahypothalamic insensitivity and/or reduced blood-brain transport. The influence of photoperiod on leptin responses and leptin transport from blood into cerebrospinal fluid (CSF) was examined in sheep. Sheep kept on *ad libitum* food for 15 wk in long days (LD) had higher voluntary food intake and lower GnRH/LH output than in short days (SD). Food intake was decreased approximately 30% after intracerebroventricular (icv) (and not iv) leptin injection, but only in SD. GnRH/LH secretion was decreased after icv (but not iv) leptin in both photoperiods. Leptin concentrations in CSF were higher in LD than SD but correlated with plasma leptin only in LD. Amounts of leptin entering CSF after iv leptin injection were greater in LD than SD. In a separate experiment, plasma (but not CSF) leptin was higher in fat than thin sheep in natural summer LD and after 5 wk in SD. CSF leptin correlated with plasma leptin in LD but not SD. CSF leptin after iv leptin injection was higher in thin than fat sheep but only in LD. Endogenous CSF to plasma concentration ratios correlated negatively with plasma concentrations, indicating decreased blood-brain transport with increased leptinemia. Therefore, icv (and not iv) leptin inhibited appetite only in SD and decreased GnRH/LH output in both photoperiods, and the proportion of circulating leptin entering CSF was higher in LD and thinner animals. Photoperiod apparently modulates intrahypothalamic leptin sensitivity of appetite, but not reproductive, regulatory pathways, whereas photoperiod and leptinemia influence leptin blood-brain transport. (*Endocrinology* 147: 4589–4598, 2006)

LEPTIN SECRETED FROM peripheral adipose tissue acts centrally within the mammalian hypothalamus to reduce appetite and promote negative energy balance (1). However, it is also clear that the typically elevated circulating leptin concentrations in obese humans fail to act in this way (2). This apparent central insensitivity or resistance to leptin may occur at or downstream of its hypothalamic receptor, or it may be due to reduced transport of leptin from the peripheral circulation into the brain. Elucidating the underlying causes of central leptin insensitivity would clearly be desirable in contributing to our understanding of the etiology of obesity.

Changes in hypothalamic sensitivity to leptin in sheep at different times of the year have been reported by two research groups (3, 4). For example, in our study, appetite suppression by intracerebroventricular (icv) injection of leptin was seen in the autumn but not the spring, consistent with an influence of photoperiod (3). Here we investigated this further by comparing responses to icv leptin in sheep kept in artificial long-day (LD) and short-day (SD) photoperiods. Leptin also acts within the hypothalamus on the reproductive neuroendocrine axis, although responses in sheep are variable (reviewed in Ref. 5). Thus, icv leptin restored LH output, and by inference hypothalamic GnRH secretion (6), in food-restricted ewes in one study (7) but not another (8) and decreased LH secretion in the adequately nourished rams in the study by Blache *et al.* (9) but had no effect in the undernourished rams in the study by Celi *et al.* (10). Furthermore, we reported a seasonal influence on the response because icv leptin stimulation of LH output in adequately fed sheep was greater in the spring than the autumn (3). This indicated that there might be photoperiod-driven changes in hypothalamic leptin sensitivity with respect to the reproductive neuroendocrine axis, and the present study aimed to elucidate this phenomenon in artificial photoperiods. Estradiol-implanted castrated male sheep were used, as in our earlier studies (3, 11), because this model provides constant physiological concentrations of gonadal steroid feedback and avoids the seasonal or photoperiod-driven changes in circulating concentrations shown by gonad-intact animals. Because estradiol feedback is critical in males as well as females (12), results from this model are relevant to both sexes. Although the model precludes changes in the amount of circulating gonadal steroid, which could influence both energy balance (13) and reproductive neuroendocrine axes (14), changes may occur in sensitivity to gonadal steroid negative feedback between the photoperiods, which are known to modulate GnRH/LH output (14).

Intracerebroventricular administration studies give pre-

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Abbreviations: AUC, Area under the curve; BCS, body condition score; CSF, cerebrospinal fluid; icv, intracerebroventricular; LD, long days; LV, lateral ventricle; SD, short days; VFI, voluntary food intake.

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cise and specific information on hypothalamic responses and sensitivity to hormones such as leptin, but they effectively circumvent putative alterations in blood-brain transport that may be equally important. It is therefore pertinent to compare responses to icv- and peripherally administered leptin within the same study and measure the amounts of peripherally circulating leptin that enter the brain in different physiological situations. The blood-cerebrospinal fluid (CSF) barrier at the choroid plexus and the blood-brain barrier at the cerebral endothelium are two major controlling sites for entry of circulating proteins, such as leptin, into the brain (15). Published studies support the concept of brain-barrier regulation of leptin entry into the central nervous system in rodents (16, 17) and, more specifically, regulated leptin transport at the blood-CSF barrier using the perfused sheep choroid plexus *in vitro* model (18). Here we examined such transport *in vivo* within the complex physiology of the whole animal. Thus, we used the icv-cannulated sheep to take samples of ventricular CSF, along with peripheral blood samples, for concurrent longitudinal measurements of leptin concentrations. Reduced transport of endogenous and exogenous leptin into brain CSF has been reported in a rat model of dietary-induced central leptin resistance (19), but transport in sheep and the putative effects of photoperiod are unknown.

The concept that reduced blood-brain transport may provide the mechanism for leptin resistance in obese humans comes from relatively few studies, which have indicated that efficiency of leptin uptake from the periphery into the brain is decreased with increased adiposity (20, 21). However, these findings are based on single samples from each subject and by necessity make the assumption that concentrations in lumbar CSF accurately represent those within the brain. The icv-cannulated sheep, similar in body size and adiposity to the human, offers the ability to study dynamic changes in blood-brain concentration relationships, with repeated samples of blood and ventricular CSF taken across longitudinal changes in physiological or nutritional status. In addition, hypothalamic responses to leptin administration via both central and peripheral routes, in terms of food intake and hypothalamo-pituitary endocrine output, are readily measurable repeatedly in the same individual. Here we demonstrate the utility of the sheep model to advance our understanding of central leptin (in)sensitivity *in vivo*.

Therefore, this study uses sheep to test the following hypotheses: that icv (intrahypothalamic) leptin inhibits appetite and stimulates GnRH/LH secretion, that the responses are regulated by photoperiod and are specific to icv-administered leptin (as opposed to peripherally administered leptin), and that blood-brain transport of leptin is modulated by photoperiod and/or leptinemia.

Materials and Methods

All experimental procedures involving animals were conducted under the authority of the United Kingdom Animals (Scientific Procedures)

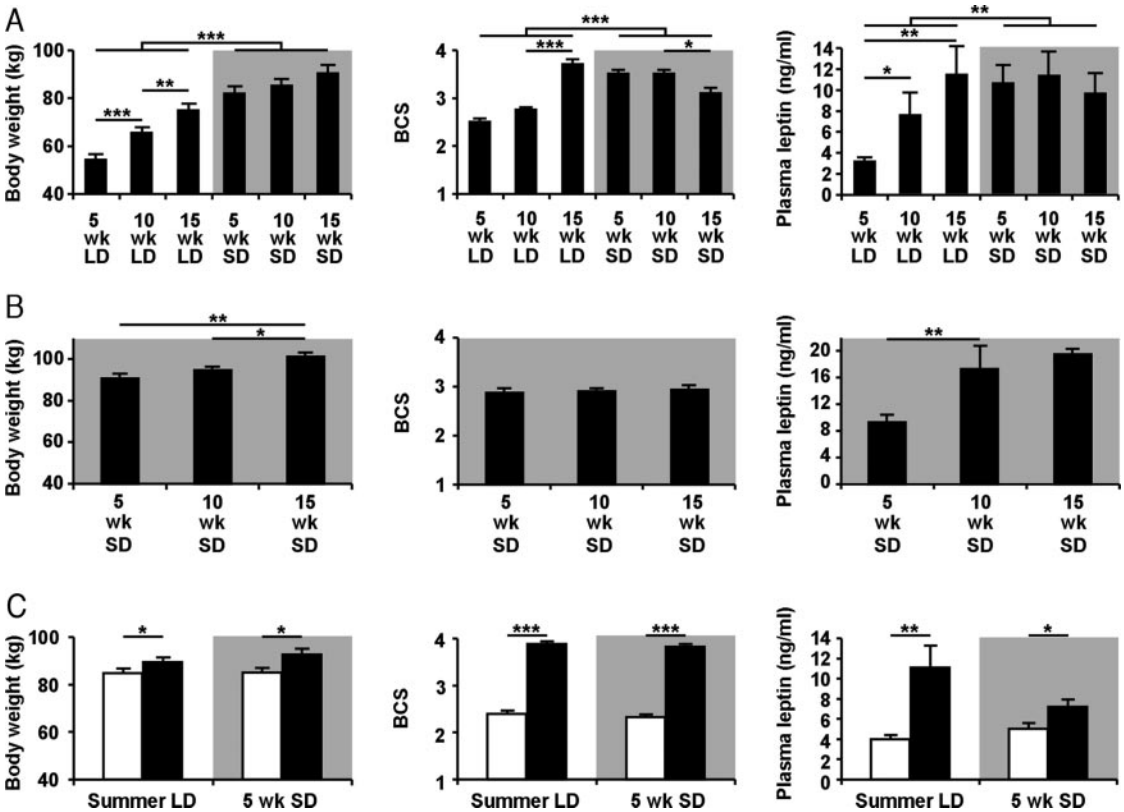

FIG. 1. Body weight, BCS (adiposity score), and endogenous plasma leptin (preprandial, preinjection morning concentrations) for sheep kept 16 wk in artificial LD (*clear background*) followed by 16 wk in SD (*gray background*) with *ad libitum* food (experiment 1a, n = 9) (A), kept 15 wk in SD with *ad libitum* food (experiment 1b, n = 9) (B), and relatively thin (*open bars*) and fat (*solid bars*) maintenance-fed sheep in natural summer LD (June) followed by 5 wk in artificial SD (experiment 2, n = 6/group) (C). Means ± SEM; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Act of 1986 and received prior approval from the local ethical review committee.

Animals

All sheep were Suffolk × Greyface adult male castrates (~1 yr old at surgery). They were housed in individual pens in natural photoperiod (in Aberdeen, UK, 57° N) or artificial photoperiod of either LD (16-h light, 8-h dark) or SD (8-h light, 16-h dark) and given a complete diet (comprising 50% chopped hay, 30% rolled barley, and 9% soybean meal, with molasses, minerals, vitamins, and trace elements) twice daily at approximately 0800 and 1600 h. Water was provided *ad libitum*.

Cannulation of the lateral ventricle (LV) and third cerebral ventricle, as previously reported by Miller *et al.* (3, 22), was undertaken in 24-h fasted sheep maintained on halothane anesthesia (2% Halothane BP; Concord Pharmaceuticals Ltd., Essex, UK). While under general anesthesia, each sheep was given two sc estradiol-containing implants made from SILASTIC tubing (Dow Corning, Midland, MI; dimension 15 × 4.8 mm) (11) designed to raise plasma estradiol concentrations to approximate physiological levels of around 1–5 pg/ml normally seen in intact luteal phase females and males (23, 24); these were measured during each experiment by RIA (25). All sheep had a minimum postoperative recovery period of 4 wk before experimentation.

Measurements were made of body weight and body condition score (BCS) once every 2 wk. BCS provides an adiposity score from assessment by palpation of the prominence and degree of cover of the spinous and transverse processes of the anterior lumbar vertebrae, scale 0 (emaciated) to 5 (obese), after Russel *et al.* (26). Voluntary food intake (VFI) of *ad libitum* food was determined daily at 0800 h by removing and measuring uneaten food.

Experiment 1

Experiment 1a. Castrated, estradiol-implanted, icv-cannulated male sheep (n = 9), that had previously been in natural spring (increasing) photoperiod, were kept for 16 wk in LD followed by 16 wk in SD. Food was restricted to maintenance (*i.e.* the daily amount required to maintain body weight) for the first 5 wk in LD but was thereafter available *ad libitum*. Initial body weight and BCS were 50.6 ± 1.35 kg and 2.5 ± 0.08, respectively, at the start of LD and 74.8 ± 2.79 kg and 3.7 ± 0.11 at the start of the SD period. Mean plasma estradiol concentration for all sheep (average of three samples taken at the start, middle, and end of the experiment) was 2.6 ± 0.33 pg/ml.

During the 2-wk periods 4–5, 9–10, and 14–15 wk in each photoperiod, each sheep received leptin (recombinant ovine leptin; PLR Ltd., Rehovot, Jerusalem, Israel) by icv route (0.5 mg in 0.1 ml 0.9% saline via third cerebral ventricle cannula) one week and iv route (5 mg in 5 ml 0.9% saline via jugular vein) the other week. Dose rates were determined from our previous experience of single icv leptin injections (3) and both theoretical calculations and a published study of iv leptin injection in sheep (27). Because the half-life of circulating leptin is characteristically short (27–29), it was calculated that plasma concentrations would be within the physiological range (~50 ng/ml) 1 h after the 5-mg iv injection. Injections were given at 0800 h, immediately before the morning feed, control (0.9% saline) on d 1, and leptin on d 2. Temporary jugular catheters were inserted for the duration of d 1 and 2 and were used to take blood samples before injection and at 15-min intervals for 8 h afterward for plasma LH analysis. In addition, when sheep received the iv injection, blood (from the jugular catheter) and CSF (from the LV cannula) were sampled before injection and at hourly intervals for 8 h for measurement of leptin concentrations.

Experiment 1b. CSF samples could not be obtained during the SD period of experiment 1a due to progressive blockage of the LV cannulae. Therefore, additional castrated, estradiol-implanted sheep prepared with two LV cannulae (n = 9), which had been in natural summer photoperiod (natural LD in June), were kept for 16 wk in artificial SD with *ad libitum* food to provide these data. Initial body weight and BCS were 78.9 ± 2.18 kg and 2.9 ± 0.11, respectively. Mean plasma estradiol concentration (average of two samples taken near the start and end of experiment) was 2.9 ± 0.22 pg/ml. During wk 5, 10, and 15, leptin (5 mg in 5 ml saline, as above) was injected iv at 0800 h, immediately before the morning feed. Blood and CSF were sampled from temporary jugular catheters and LV cannulae, respectively, before iv leptin injection and at hourly intervals over 8 h for leptin analysis.

Experiment 2

Castrated, estradiol-implanted sheep prepared with icv cannulae, with initial body weight and BCS of 84.9 ± 1.87 kg and 2.4 ± 0.06 (thin) and 89.4 ± 2.14 kg and 3.8 ± 0.05 (fat), respectively, that had been in natural photoperiod were used in the summer (*i.e.* natural LD in June). They were given maintenance amounts of food and were transferred to artificial SD for 5 wk (n = 6/group). Mean plasma estradiol concentration (average of two samples taken at the start and end of experiment) was 2.1 ± 0.25 pg/ml.

On 1 d in natural LD photoperiod and again after 5 wk in SD, leptin (5 mg in 5 ml saline, as above) was injected iv at 0800 h, immediately before the morning feed. Blood and CSF were sampled from temporary jugular catheters and LV cannulae, respectively, before iv leptin injection and at hourly intervals over 8 h for leptin analysis.

Leptin RIA

Leptin concentrations were determined in duplicate for plasma and CSF by homologous RIA (30). The leptin standard in the RIA was the same leptin preparation used for injecting the animals. Samples from each experiment were analyzed in a single assay run. Intra- and interassay coefficients of variation averaged 3 and 11%, respectively, with detection limit 0.05 ng/ml.

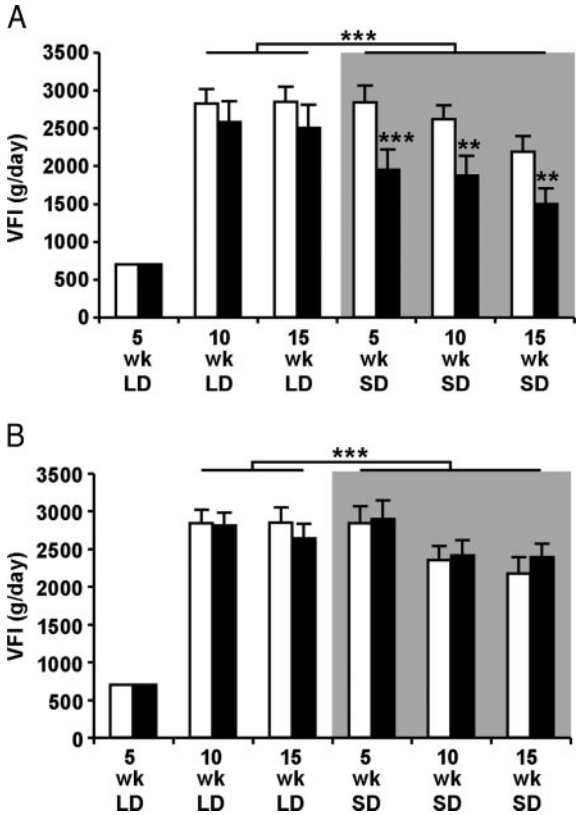

FIG. 2. Twenty-four hour VFI by sheep after icv leptin injection (0.5 mg in 0.1 ml 0.9% saline) (A) or iv leptin injection (5 mg in 5 ml 0.9% saline) (*solid bars*) (B) at 10 and 15 wk in LD photoperiod (*clear background*) and at 5, 10, and 15 wk in SD photoperiod (*gray background*) (experiment 1a, n = 9). Control treatment was 0.9% saline injection (*open bars*). Food intake at 5 wk LD was not *ad libitum* and was therefore not included in the statistical analysis. Means ± SEM; **, $P < 0.01$; ***, $P < 0.001$.

LH RIA

LH concentrations were determined in duplicate aliquots of plasma samples by RIA (31) using reagents provided by the National Institute of Digestive and Kidney Disorders (Rockville, MD) and expressed in terms of the reference standard NIDDK-oLH-1-2. Intra- and interassay coefficients of variation averaged 5 and 10%, respectively, and the detection limit was 0.05 ng/ml.

Statistical analysis

In experiment 1a, data during each defined 2-wk time point were collated by injection route, and for the sake of clarity these time points are rounded up and referred to as 5, 10, and 15 wk. LH concentration data from the 8-h serial plasma samples were analyzed using the Pulsefit program (supplied by R. Kushler and M. Brown, University of Michigan, Ann Arbor, MI) to provide pulse frequency, pulse amplitude, and mean concentration values for each individual profile. This algorithm is based on a nonlinear statistical model incorporating exponential decay between distinct pulses (32). Pulses identified by the algorithm are screened to include only those where the difference between the peak and previous nadir exceed the assay sensitivity. VFI, mean LH, LH pulse frequency, and LH pulse amplitude data were examined by ANOVA (MINITAB 14; Minitab Inc., State College, PA) using the general linear model with individual sheep, time, photoperiod and treatment, and their interaction, as specified terms.

In experiments 1a, 1b, and 2, ANOVA (general linear model or two-way; MINITAB 14) was used to compare leptin concentrations between treatments and photoperiods, and linear regression analysis (MINITAB 14) was used to explore relationships between CSF and plasma leptin concentrations.

Results are presented as group means ± SEM and differences are deemed significant at $P < 0.05$.

Results

Body weight increased throughout experiment 1a with values therefore higher in SD than LD ($P < 0.001$), BCS increased in LD and decreased at the last time point in SD, with values therefore higher in SD than LD ($P < 0.001$), whereas plasma leptin increased in LD and remained unchanged in subsequent SD, with values therefore higher in SD than LD ($P < 0.01$) (Fig. 1A). In experiment 1b (SD only), body weight and plasma leptin increased (both $P < 0.01$), whereas BCS remained constant (Fig. 1B). Body weight ($P < 0.05$), BCS ($P < 0.001$), and plasma leptin ($P < 0.05$–0.01) were lower in the thin group than the fat group of experiment 2 in natural summer LD and had not changed significantly after 5 wk in artificial SD (Fig. 1C).

Food intake (experiment 1a)

At 5 wk LD food intake remained restricted to maintenance levels, with leptin treatment having no effect (Fig. 2, A and B); data from this time point were therefore excluded from the analysis of VFI. When VFI data were analyzed together for icv injection days (control and leptin), daily VFI was higher in LD than SD ($F_{1,74} = 11.39$, $P < 0.001$) and higher after control than leptin injection ($F_{1,74} = 15.99$, $P < 0.001$), with significant interanimal variation ($F_{8,74} = 8.32$, $P < 0.001$) (Fig. 2A). There was no overall interaction with time, but examination of the separate time points revealed that VFI was decreased by leptin given icv only in SD, by approximately 30% on all three occasions ($F_{1,40} = 22.15$, $P < 0.001$). Control intake at 15 wk LD and 5 wk SD was not significantly different. When all VFI data were analyzed together for iv injection days, VFI was higher in LD than SD ($F_{1,74} = 18.97$, $P < 0.001$),and there was significant interanimal variation ($F_{8,74} = 9.80$, $P < 0.001$), as above but no effect of leptin *vs.* control injection, no interaction with time, and no difference in control intake between 15 wk LD and 5 wk SD (Fig. 2B). Therefore, daily VFI was higher in LD than SD, and icv leptin decreased VFI only in SD, whereas iv leptin had no effect on intake in either photoperiod.

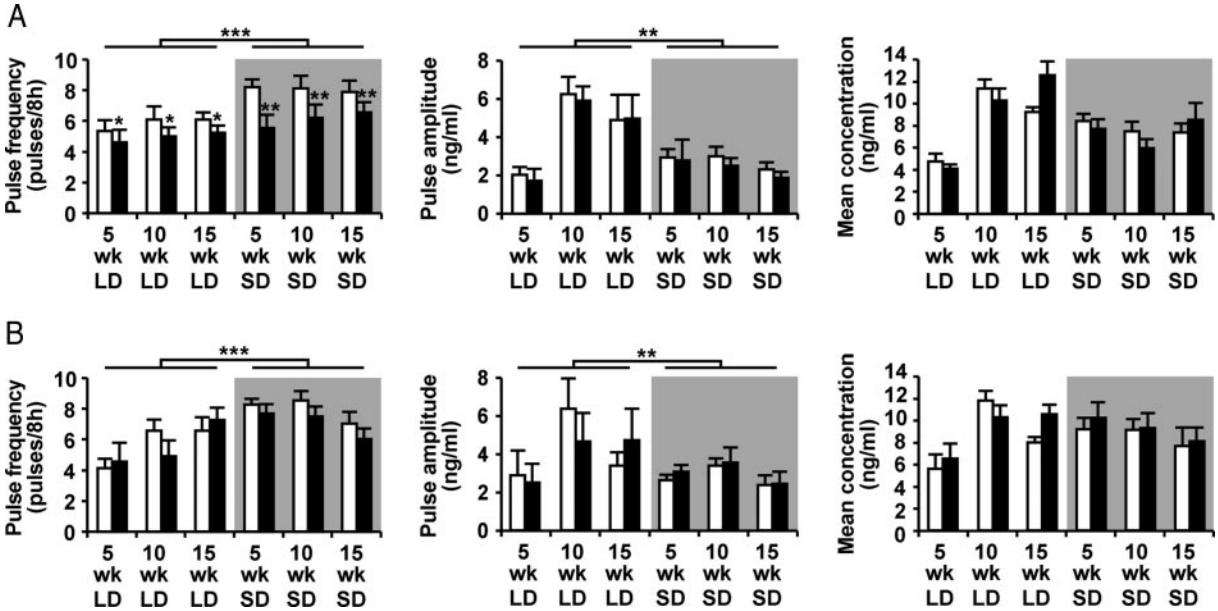

FIG. 3. Pulsatile LH secretory parameters (pulse frequency, pulse amplitude, and mean concentration) over 8 h in sheep after icv leptin injection (0.5 mg in 0.1 ml 0.9% saline) (A) or iv leptin injection (5 mg in 5 ml 0.9% saline) (B) at 5, 10, or 15 wk in LD (*clear background*) or SD (*gray background*) photoperiod (experiment 1a, n = 9) (*solid bars*). Control treatment was 0.9% saline injection (*open bars*). Control *vs.* leptin data were compared overall and within photoperiods by ANOVA. Means ± SEM; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

LH secretion (experiment 1a)

ANOVA for each LH secretory parameter for all data together on icv injection days revealed significant effects of photoperiod on pulse frequency ($F_{1,89} = 11.79$, $P < 0.001$) and pulse amplitude ($F_{1,89} = 9.40$, $P < 0.01$) but not on mean LH concentrations (Fig. 3A). Interanimal variation was significant for mean LH ($F_{8,90} = 8.75$, $P < 0.001$) and pulse frequency ($F_{8,89} = 2.09$, $P < 0.05$), and there were significant effects of time on mean LH ($F_{2,90} = 17.23$, $P < 0.001$) and pulse amplitude ($F_{2,89} = 9.97$, $P < 0.001$). Similarly, ANOVA for all data together on iv injection days revealed significant effects of photoperiod on pulse frequency ($F_{1,90} = 17.02$, $P < 0.001$) and pulse amplitude ($F_{1,90} = 8.89$, $P < 0.01$) but not on mean LH concentrations (Fig. 3B). Interanimal variation was significant for mean LH ($F_{8,90} = 4.94$, $P < 0.001$) and pulse amplitude ($F_{8,90} = 4.51$, $P < 0.001$), and there were significant effects of time on mean LH ($F_{2,90} = 4.78$, $P < 0.01$) and pulse amplitude ($F_{2,90} = 3.86$, $P < 0.05$). There was an overall approximately 20% decrease in LH pulse frequency after icv leptin in both photoperiods at all time points ($F_{1,89} = 7.65$, $P < 0.01$), with no interaction, but iv leptin had no significant effect in either photoperiod. Neither icv nor iv leptin significantly affected LH pulse amplitude or mean LH. Thus, LH pulse frequency was higher in SD than LD, and pulse amplitude was higher in LD than in SD; icv leptin decreased LH pulse frequency irrespective of photoperiod, and iv leptin had no effect (Fig. 3, A and B).

Plasma and CSF leptin concentrations (experiments 1a and 1b)

Endogenous leptin concentrations in CSF were higher in the LD of experiment 1a than the SD of experiment 1b, and they correlated positively with concurrent plasma values in LD ($r^2 = 0.57$ and significance of linear regression $P < 0.001$) but not in SD (Fig. 4A). Thus, the CSF to plasma concentration ratio was constantly 0.16 across the range of plasma concentrations in LD, whereas this ratio in SD was both lower (average 0.02) and negatively correlated with corresponding plasma concentrations ($r^2 = 0.59$, $P < 0.001$; Fig. 4B).

Plasma leptin concentrations increased sharply after iv leptin injection, peaking at 1 h and decreasing to endogenous values after 7 h, with similar profiles seen at all time points in both photoperiods (Fig. 5, A and B); however, although simultaneous CSF leptin concentrations also peaked at 1 h and decayed thereafter, these profiles were visibly muted in SD, compared with LD (Fig. 5, A and B). Thus, the area under the CSF leptin response curve (AUC) was significantly greater in LD than SD at all time points ($F_{1,25} = 18.72$, $P < 0.001$) (Fig. 6A). AUC was negatively correlated with endogenous (preinjection) plasma leptin concentration in the LD ($r^2 = 0.27$, $P < 0.05$) but not SD photoperiod (Fig. 6B).

Plasma and CSF leptin concentrations (experiment 2)

Endogenous plasma leptin concentrations were higher in the fat group than the thin group ($F_{1,20} = 15.44$, $P < 0.001$) but not different between the photoperiods ($F_{1,20} = 1.48$) (Fig. 1C). Conversely, CSF concentrations were significantly different between the photoperiods ($F_{1,20} = 21.87$, $P < 0.001$) but not between fat and thin groups ($F_{1,20} = 3.02$) (Fig. 7A). The CSF to plasma concentration ratio was significantly different between photoperiods ($F_{1,20} = 15.99$, $P < 0.001$) and between fat and thin groups ($F_{1,20} = 4.42$, $P < 0.05$), with significant interaction ($F_{1,20} = 7.64$, $P < 0.01$) (Fig. 7A). Thus, plasma leptin was higher in fat than thin sheep, CSF leptin was higher in summer LD than SD, and the CSF to plasma leptin concentration ratio was highest in thin sheep in summer LD. CSF leptin concentrations were positively correlated with simultaneous plasma concentrations in LD ($r^2 = 0.39$ and significance of linear regression $P < 0.05$) but not in SD (Fig. 7B); and the CSF to plasma ratio was negatively correlated with plasma concentrations in LD ($r^2 = 0.33$, $P < 0.05$) but not SD (Fig. 7C).

Plasma leptin profiles after iv leptin were similar in both fat and thin groups in summer LD and SD, with a peak at 1 h after injection and decay thereafter (Fig. 8A). Corresponding CSF leptin profiles also peaked at 1 h, and this peak was higher in thin than fat sheep in summer LD ($P < 0.05$) but not after 5 wk in SD (Fig. 8B). However, AUC for

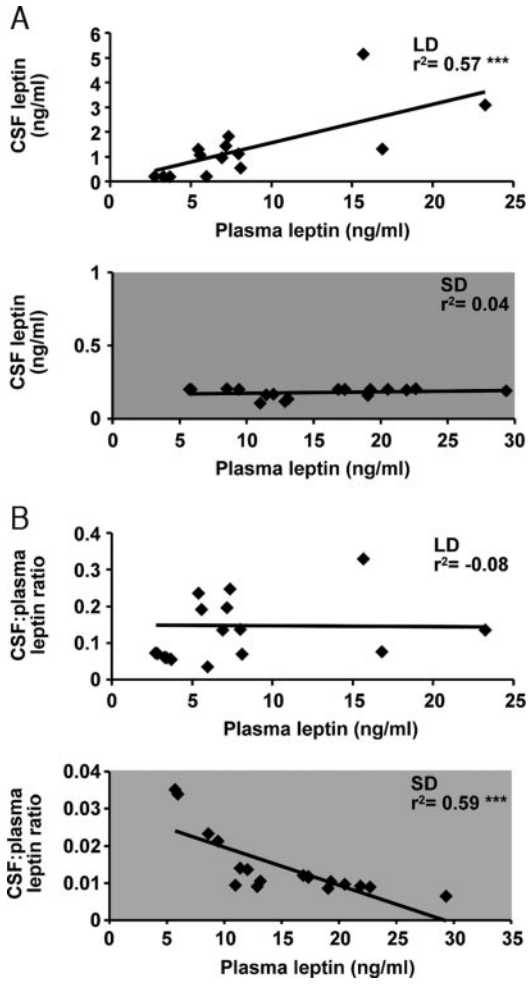

FIG. 4. Correlation (linear regression) between endogenous concentrations of leptin in CSF and plasma (preprandial, preinjection morning samples) (A) and between CSF to plasma concentration ratio and endogenous plasma concentrations (B) for sheep at 5, 10, and 15 wk in LD (*clear background*, experiment 1a, n = 9) or SD photoperiod (*gray background*, experiment 1b, n = 9). ***, $P < 0.001$.

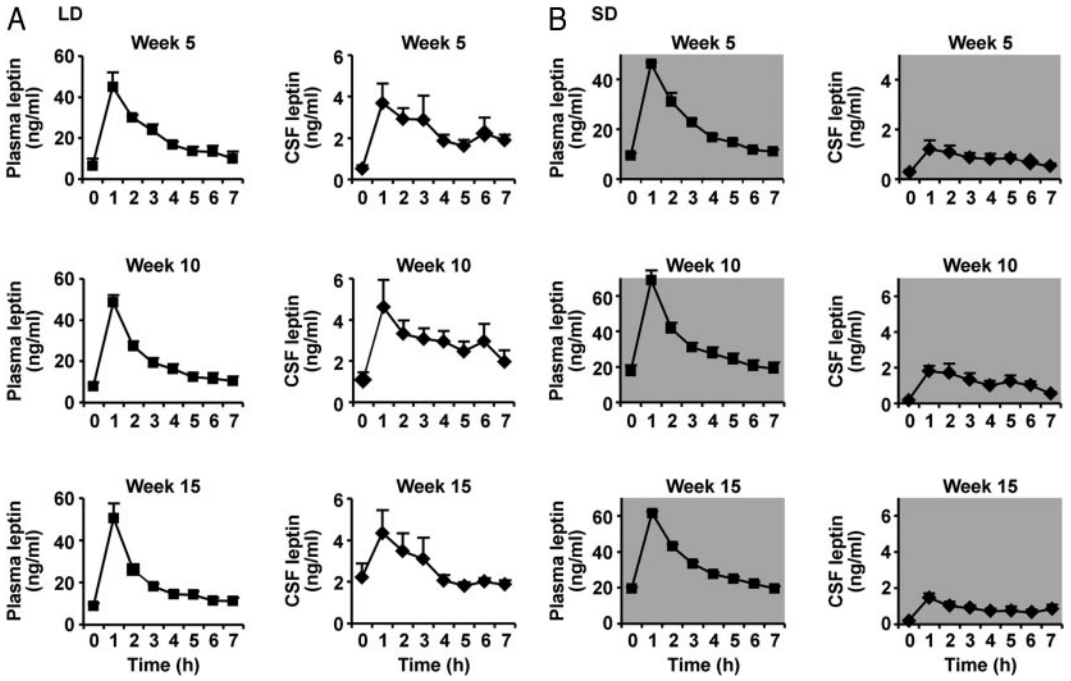

FIG. 5. Plasma and CSF leptin concentration profiles in sheep before (time 0 h) and after iv (5 mg) leptin injection at 5, 10, or 15 wk (A) in LD (*clear background*) photoperiod (experiment 1a, n = 9) and (B) in SD (*gray background*) photoperiod (experiment 1b, n = 9). Means ± SEM.

CSF leptin was unaffected by photoperiod or group, and there was no correlation between AUC and endogenous (preinjection) plasma leptin concentration in either photoperiod (Fig. 8C).

Discussion

These studies have demonstrated in sheep that leptin delivered icv (and not iv) inhibited appetite, but only in SD, and decreased GnRH/LH pulsatile output in both photoperiods. Proportional transfer of leptin from blood to CSF was consistently greater in LD than SD, and relative transfer also tended to be increased at lower plasma concentrations. Therefore, photoperiod apparently influences hypothalamic responses to leptin with respect to the appetite axis and not the reproductive neuroendocrine axis, and blood-brain transport of leptin is influenced by photoperiod and leptinemia.

Stronger evidence for the influence of photoperiod *per se* would have been obtained had the experimental design also included a shift from SD to LD. Nonetheless, the present results obtained in animals shifted from LD to SD days are clear. It is particularly striking that the changes in intake response to icv leptin and in leptin blood-brain transport were already established at the first time point studied in SD, before measurable photoperiod-driven changes in nutritional status (food intake and adiposity). Potential confounding by longitudinal effects and/or relative stage of maturity was addressed by the inclusion of time as a factor in the statistical analysis; this was probably minimal because no interaction was detected between treatment effects and time.

The results support the hypothesis that leptin inhibits appetite in sheep (in agreement with previous studies, *e.g.* Refs. 3, 4, 9) and that the response is both specific to intrahypothalamic (icv administered) leptin and influenced by photoperiod. Thus, against the background of characteristically lower appetite drive (VFI) in SD than LD sheep (*e.g.* reviewed in Ref. 33), icv leptin decreased VFI only in SD. Although a higher dose of leptin may have been effective in LD, the present data nonetheless support our earlier conclusion that the observed seasonal difference in appetite response to icv leptin was an effect of photoperiod, with sensitivity greater in SD than LD (3). This indicates that hypothalamic appetite regulatory mechanisms are less sensitive to inhibitory input during LD than during SD, possibly due to a shift in leptin receptor sensitivity. The difference was unlikely to be attributable to changes in gonadal steroid concentration (13) because steroid-clamped castrates were used or to changes in sensitivity to gonadal steroid feedback because Clarke *et al.* (4) report a seasonal influence on the anorectic effects of icv leptin in gonadectomized sheep without steroid replacement.

Photoperiodic modulation of leptin-induced body weight (or fat) loss has also been reported in seasonal rodents, with sensitivity to leptin higher in SD than LD in Siberian hamsters (34, 35) and field voles (36). However, leptin in these rodent studies was given peripherally, and it is not clear the extent to which differences at the level of intrahypothalamic signaling contributed to the observed responses. In the present sheep study, in contrast to icv-administered leptin, iv-administered leptin had no effect on appetite. This indicates that leptin does have to enter the brain first for its actions on hypothalamic appetite drive, despite the reported presence in rodent brain of leptin receptors in cells situated outside the blood-brain barrier in the ventral region of the hypothalamic arcuate nucleus (37). Morrison *et al.* (38) also reported the lack of a hypophagic effect of leptin delivered

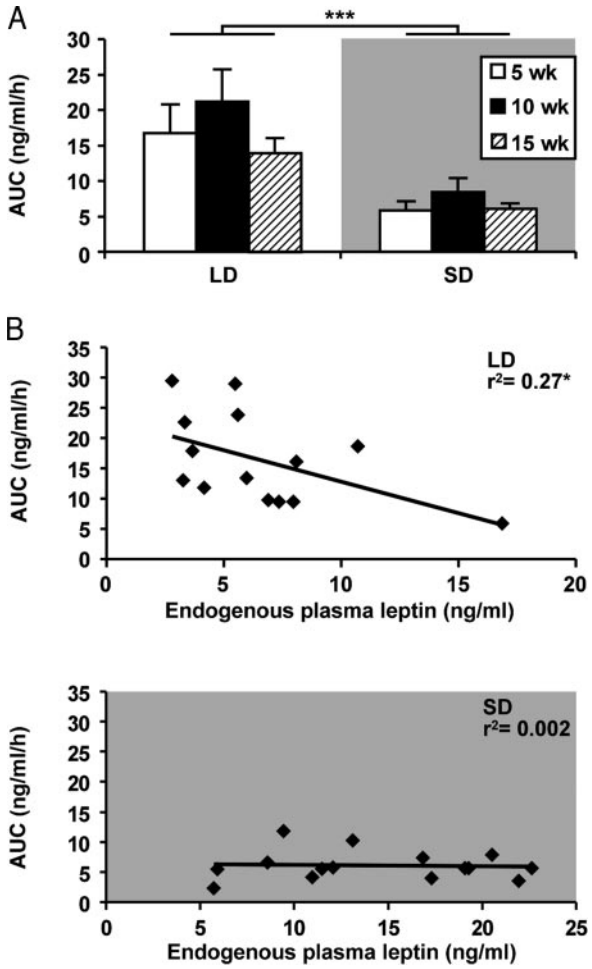

FIG. 6. A, CSF leptin concentrations, expressed as the 8-h response AUC after iv (5 mg) leptin injection at 5, 10, or 15 wk in LD photoperiod (*clear background*; experiment 1a, n = 9) and SD photoperiod (*gray background*; experiment 1b, n = 9) (means ± SEM). B, Correlation (linear regression) between AUC (at 5, 10, and 15 wk) and endogenous plasma leptin concentration (preinjection morning sample) for sheep in LD (experiment 1a) and SD (experiment 1b). *, $P < 0.05$; ***, $P < 0.001$.

iv to well-fed lambs, in contrast to the effects of leptin delivered icv in an earlier study (8), again suggesting that leptin entry to the brain may be an important site of leptin resistance. In the present study, the lack of effect of iv leptin on appetite in SD may have been attributable to the reduced blood-brain leptin transport in this photoperiod; this would have prevented sufficient leptin accessing the hypothalamus at a time when it was apparently primed to be leptin sensitive according to the present hypophagic response to leptin delivered icv in SD. Conversely, the lack of effect of iv leptin on appetite in LD occurred despite increased amounts of leptin accessing the hypothalamus because the hypothalamic targets were apparently relatively leptin insensitive at this time, according to the present lack of response to icv leptin in LD.

In contrast to leptin actions on appetite, the results do not support the hypothesis that leptin stimulates the reproductive neuroendocrine axis or that this effect is influenced by photoperiod. Rather, leptin appeared to decrease reproductive neuroendocrine output, and this effect was specific to icv delivery. Sheep are seasonal breeders, with reproductive activity stimulated in SD, driven by the increased output frequency of pulsatile GnRH (*e.g.* reviewed in Ref. 33). Thus, in the present animals, photoperiod characteristically influenced pituitary LH secretion, and by inference hypothalamic GnRH output (6), with LH pulse frequency higher in SD and pulse amplitude higher in LD. Against this background, the only significant effect of exogenous leptin was to decrease LH pulse frequency, irrespective of photoperiod, in response to icv leptin but not when the leptin was given iv. From this, our earlier observation of a seasonal difference in response to icv leptin between autumn and spring cannot be explained solely by differences in photoperiod (3). Furthermore, icv leptin stimulated GnRH/LH output in the earlier study and inhibited GnRH/LH in the present study.

This discrepancy may be attributable to the fact that the sheep in the earlier study were restricted to maintenance feeding, whereas those in the present study were fed *ad libitum* (apart from the first time point when they were maintenance fed and icv leptin had no effect). Indeed reports of leptin stimulation of GnRH/LH in sheep are largely limited to food-restricted animals [reviewed by Adam *et al.* (5)] that have low frequency GnRH/LH secretion. Conversely, in the well-fed rams in the study by Blache *et al.* (9), with high frequency GnRH/LH secretion, icv leptin inhibited LH pulse frequency, as seen in the present *ad libitum*-fed sheep. In both studies, the decrease in LH may have been a secondary effect of the leptin-induced hypophagia and/or indicative that leptin is permissive to reproductive function only within a range of concentrations, with both excess leptin and insufficient leptin detrimental to reproductive function. Others have also reported no effect of peripherally administered iv leptin on LH secretion in undernourished lambs (38), lending further support to the intrahypothalamic specificity of any effect of leptin on GnRH/LH. However, the nature of leptin's action on the reproductive neuroendocrine axis remains equivocal.

Finally, the present data support the hypothesis that photoperiod regulates blood-brain transport of leptin in sheep. Endogenous circulating plasma leptin concentrations generally reflected body size and adipose status in the *ad libitum*-fed sheep of experiment 1 and the fat *vs.* thin maintenance-fed sheep of experiment 2. However, there was an additional dampening effect of the restricted food intake in experiment 2 (30, 39); hence, although fat sheep in experiment 2 had higher BCS than the sheep in experiment 1, their plasma leptin concentrations were lower. Thus, the experimental paradigms allowed examination of leptin blood-brain CSF entry in relation to both photoperiod and background leptinemia. The endogenous concentration of leptin in CSF was a greater proportion of the amount in the circulating blood from which it came in LD than SD, over a similar range of plasma leptin concentrations and across all time points in both experiments 1 and 2.

Photoperiodic regulation of blood-brain transport is not without precedent because it has also been reported for reproductive steroid hormones in sheep (40, 41), although not for peptide hormones like leptin. Thus, sex steroid access to the brain is apparently increased during LD (41), consistent with our present findings for leptin. Changes in circulating sex steroid concentrations are unlikely to be the cause of

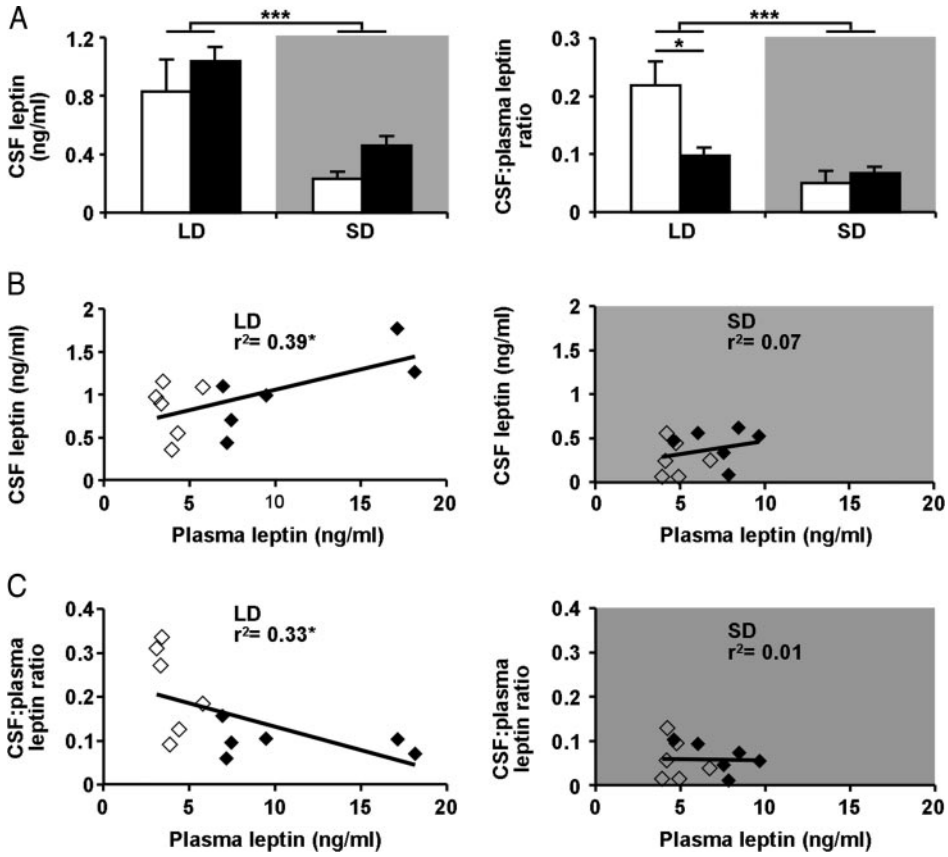

FIG. 7. A, Endogenous CSF concentrations of leptin and CSF to plasma concentration ratios in thin (*open bars*) and fat (*solid bars*) maintenance-fed sheep in natural summer LD (in June, *clear background*) and after 5 wk in artificial SD (*gray background*) (means ± SEM, experiment 2, n = 6/group). B, Correlation (linear regression) between CSF and simultaneous plasma leptin concentrations for all sheep (thin, *open symbols*; fat, *solid symbols*) in LD and SD. C, Correlation (linear regression) between CSF to plasma concentration ratio and plasma concentrations for all sheep in LD and in SD. *, $P < 0.05$; ***, $P < 0.001$.

altered leptin transport because levels were clamped in the estradiol-implanted castrate model used here. However, whereas peripheral estradiol levels may not be changing, photoperiod could regulate its blood-brain transport (41), resulting in increased brain estradiol levels in LD that could in turn regulate leptin blood-brain transport. It is pertinent that a study of ovariectomized mice concluded that estrogens are required to facilitate leptin entry into the brain (42). Here there was no evidence for further changes in leptin blood-brain transport with increased duration in each photoperiod and the photoperiod-driven change was apparently in place within 5 wk. Conclusions drawn from endogenous CSF and plasma leptin concentration relationships were supported by similar findings after peripheral leptin administration. Thus, more of the exogenous leptin entered the CSF in LD than SD, despite virtually identical induced plasma leptin profiles, at all time points in experiment 1 and for the thin sheep in experiment 2.

Therefore, in the contrasting photoperiod paradigm, it appears that increased hypothalamic sensitivity to leptin occurs at a time when blood-CSF leptin transport is decreased in SD photoperiod and vice versa in LD photoperiod. It is tempting to speculate cause and effect, *i.e.* that the increased amounts of leptin able to access the hypothalamic CSF in LD may contribute to the saturation or down-regulation of the leptin receptor or postreceptor signaling pathway; and conversely, the leptin receptor and/or postreceptor pathways are able to remain sensitized when exposed to the decreased amounts of leptin in CSF in SD. Alternatively, photoperiod (presumably transduced by melatonin or secondary messenger, *e.g.* reviewed in Ref. 43), may inhibit leptin brain entry at the blood-CSF barrier in SD to prevent its intrahypothalamic anorectic actions when appetite and energy balance are at a seasonal low (33). Inhibition of brain uptake of leptin would be unnecessary in LD when the seasonally maximal appetite drive apparently cannot be overridden by intrahypothalamic actions of leptin. This scenario lends weight to the concept that leptin feedback has evolved as a mechanism to protect from critical negative energy balance rather than prevent obesity.

Reduced blood-brain transport of leptin has been directly associated with hyperleptinemia in rodent models (16). We therefore further explored relationships between endogenous CSF leptin concentration expressed as proportion of the simultaneous plasma value and the plasma value itself. In experiment 1, at the overall higher CSF to plasma leptin ratio in LD (0.16), there was no evidence that the ratio was affected by leptinemia, but the overall lower ratio in SD was inversely related to plasma concentrations (~0.03 down to 0.01 across plasma values 5 to 30 ng/ml). In experiment 2, the overall higher ratio in LD was inversely correlated with plasma concentrations (~0.30 down to 0.10 across plasma values 3 to 18 ng/ml) whereas the overall lower ratio in SD (0.05) did not correlate with plasma concentrations. However, in experiment 1, the amount of leptin entering the CSF after exogenous peripheral administration, as judged by the CSF leptin concentration response AUC, was inversely related to the endogenous preinjection plasma concentration in LD but not SD. Furthermore, in the LD of experiment 2, the peak CSF leptin value achieved after exogenous peripheral adminis-

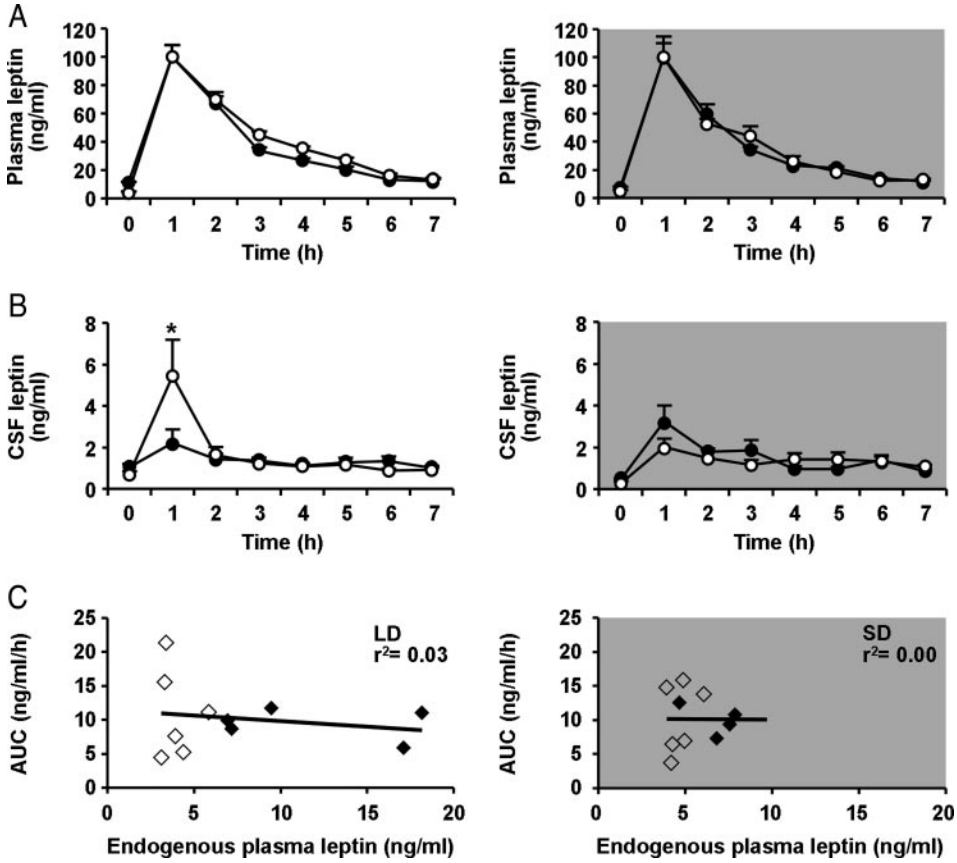

FIG. 8. Plasma leptin concentration profiles (A) and CSF leptin concentration profiles (B) before (time 0 h) and after iv (5 mg) leptin injection in thin (*open circles*) and fat (*solid circles*) maintenance-fed sheep in natural summer LD (June, *clear background*) and after 5 wk in SD (*gray background*) (means ± SEM, experiment 2, n = 6/group); and (C) Correlation (linear regression) between CSF leptin concentration response (AUC) and endogenous plasma leptin concentration (preinjection morning sample) for all sheep (thin, *open symbols*; fat, *solid symbols*) in LD and SD. *, $P < 0.05$.

tration was higher in relatively thin sheep with low endogenous circulating leptin than relatively fat sheep with higher endogenous circulating leptin. Thus, the maximum proportional transfer of leptin from blood to CSF was seen in thinner sheep in LD. Altogether, these data lend some support to the hypothesis that the efficiency of blood-brain transfer of leptin is influenced by leptinemia, being increased when circulating levels are low and decreased when circulating leptin is high.

Therefore, these experiments have provided *in vivo* evidence in sheep for photoperiod-driven changes in intrahypothalamic leptin sensitivity shown by the appetite regulatory axis but not the reproductive neuroendocrine axis and for effects of photoperiod and leptinemia on the efficiency of leptin blood-brain transport.

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