Seasonal effects of central leptin infusion on secretion of melatonin and prolactin and on *SOCS-3* gene expression in ewes

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Abstract

Recent studies have demonstrated photoperiodic changes in leptin sensitivity of seasonal mammals. Herein, we examined the interaction of season (long days (LD) versus short days (SD)) and recombinant ovine leptin (roleptin) on secretion of melatonin and prolactin (PRL) and on mRNA expression of suppressor of cytokine signaling-3 (*SOCS-3*) in the medial basal hypothalamus (MBH) in sheep. Twenty-four Polish Longwool ewes, surgically fitted with third ventricle (IIIIV) cannulas, were utilized in a replicated switchback design involving 12 ewes per season. Within-season and replicate ewes were assigned randomly to one of three treatments (four ewes/treatment) and infused centrally three times at 0, 1 and 2 h beginning at sunset. Treatments were 1) control, Ringer–Locke buffer; 2) L1, roleptin, 0.5 µg/kg BW; and 3) L2, roleptin, 1.0 µg/kg BW. Jugular blood samples were collected at 15-min intervals beginning immediately before the start of infusions and continued for 6 h. At the end of blood sampling, a washout period of at least 3 days elapsed before ewes were re-randomized and treated with one of the treatments described above (four

ewes/treatment). Ewes were then killed and brains were collected for MBH processing. Leptin treatments increased ($P < 0.001$) circulating leptin concentrations compared with controls during both seasons in a dose-dependent manner. Overall, mean plasma concentrations of melatonin were greater ($P < 0.001$) during LD than SD. However, leptin treatments increased melatonin concentrations during SD in a dose-dependent manner and decreased it during LD. Similarly, plasma concentrations of PRL were greater ($P < 0.001$) during LD than SD. However, unlike changes in melatonin, circulating PRL decreased ($P < 0.001$) in response to leptin during LD. Semi-quantitative PCR revealed that leptin increased ($P < 0.001$) *SOCS-3* expression in the MBH region during LD in a dose-dependent manner. Data provide evidence that secretion of photoperiodic hormones such as melatonin and PRL are inversely regulated by leptin during SD and LD. However, the increase in expression of *SOCS-3* in the MBH during LD compared with SD fails to fully explain these effects.

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Introduction

In temperate latitudes, sheep are seasonal breeders whose reproductive activity is controlled mainly by photoperiod. Nocturnal secretion of pineal-derived melatonin provides information about day length, but neither the target sites for its action in the brain nor the neuropeptide circuits engaged by the melatonin signal are well-defined (Lincoln *et al.* 2006).

Exposure to short days (SD) decreases leptin gene expression and hormone release in adipose tissues of Siberian hamsters, indicating that leptin may be involved in photoperiod-dependent seasonal adaptations of mammals, independent of food deprivation or overfeeding (Klingenspor *et al.* 1996, 2000). Recently, our laboratory has shown that recombinant ovine leptin (roleptin) is able to modulate melatonin release by ovine

pineal gland explants *in vitro*, and that this effect is seasonally dependent (Zieba *et al.* 2007). In seasonal mammals, melatonin also regulates photoperiodic changes in plasma concentrations of prolactin (PRL) through a melatonin-dependent oscillator located in the pars tuberalis. However, information on the effect of leptin on PRL secretion is meager and conflicting. PRL is an essential hormone for mammary gland development and milk production, and modulates adipose tissue lipid metabolism in adipocytes (Symonds & Clarke 1998). However, leptin receptors (LR) on lactotrophs are scarce, and leptin infusion into the arcuate nucleus (ARC) and median eminence stimulates PRL secretion. This suggests a control pathway mediated by hypothalamic neurons (Watanobe & Habu 2002), although direct stimulation of PRL secretion from pituitary explants is possible (Accorsi *et al.* 2007).

If the major control of seasonal changes to leptin sensitivity is at the hypothalamic level, how is this effect mediated. Although several potential mechanisms to account for this state have been proposed (Levin *et al.* 2004, Münzberg *et al.* 2005), the one receiving the most attention has been the inhibition of intracellular leptin signaling by suppressors of cytokine signaling-3 (SOCS-3), which can occur within leptin's primary targets in the ARC (Adam & Mercer 2004). Studies in the Siberian hamster (*Phodopus sungorus*, Tups *et al.* 2004) have demonstrated that reduced SOCS-3 activity during short SD contributes to the increased sensitivity to leptin and, conversely, that increased activity of SOCS-3 signaling contributes to the relative leptin insensitivity seen in long days (LD). Changes in hypothalamic sensitivity to leptin in sheep at different times of the year have been reported by two re-search groups (Clarke *et al.* 2001, Adam *et al.* 2003, 2006). However, those studies mainly investigated the photoperiodic regulation of appetite and reproductive axes.

The work reported herein was designed to determine how leptin modulates melatonin and PRL secretion in mature sheep during natural LD and SD in order to better understand the interplay between three hormones known to be under the influence of photoperiod. The second aim was to accumulate preliminary information on the relationship between SOCS-3 gene expression in the MBH and leptin-mediated effects.

Materials and Methods

All animal-related procedures used in these studies were approved by the Local Agricultural Animal Care and Use Committee of Krakow (protocol no. 25/OP/2005).

Studies were carried out at the Experiment Station in the Department of Sheep and Goat Breeding, Agricultural University of Krakow (longitude 19°57' E, latitude 50°04' N). Twenty-four female Polish Longwool sheep, a breed that exhibits strong reproductive seasonality, were used. Animals were 2–3 years of age, weighed 60 ± 5 kg, and were housed in individual pens in natural photoperiodic and thermoperiodic conditions. Sheep were in good body condition (BCS = 3) on a 1–5 scale (Russel *et al.* 1969), and were fed twice daily at 0700 and 1600 h a diet formulated to provide 100% of the National Research Institute of Animal Production recommendations for maintenance (Norms 1993). Water was available *ad libitum*.

Neuroendocrine model

In a previous experiment reported from this laboratory, we demonstrated that the ability of roleptin to modulate pineal gland melatonin secretion was dependent on day length when tissues were collected from sexually mature female sheep kept under natural photoperiod (Zieba *et al.* 2007). In that experimental model, the secretion of melatonin from ovine pineal glands was negatively responsive to leptin during LD, whereas leptin-stimulated melatonin secretion during

SD (Zieba *et al.* 2007). Therefore, for the present study, we measured melatonin secretion directly after roleptin was infused into the third ventricle (IIIV).

Procedures

Twenty-four female mature sheep fasted for 24 h were fitted surgically with IIIV cannulas according to the methodology of Traczyk & Przekop (1963). The location and function of the cannulas were verified by the continuous flow of cerebrospinal fluid (CSF). A period of at least 3 weeks was allowed for sheep to recover from neurosurgery.

Before the study began, ewes were placed frequently in individual carts to familiarize them with the experimental conditions. All experiments started at sunset with 12 ewes selected randomly during LD (March, April & May) and an additional 12 ewes during SD (September, October, and November). During LD, ewes were anovulatory and expressed no signs of estrus. During SD, estrous cycles were synchronized using a 14-day treatment with intravaginal progesterone-impregnated sponges (40 mg fluorogestone acetate, FGA, Chronogest; Intervet International, Boxmeer, The Netherlands). Ewes were also injected with a single dose of 500 IU of pregnant mares serum gonadotropin i.m. (Serogonadotropin, Biowet, Drwalew, Poland) on the day of sponge removal. Estrous detection was performed twice daily with an adult ram equipped with an apron. Ewes were presented individually to the male. Estrus was defined as an acceptance of mounting. Experiments were performed when ewes were in the mid-luteal phase (days 7–10) of the oestrous cycle.

In the morning of the day of each experiment, sheep were fitted with jugular catheters for intensive blood sampling. Polyethylene tubing (70 mm; 0.58 i.d., 0.96 mm o.d.; Intramedic Clay Adams Brand, Becton Dickinson, Sparks, MD, USA) was inserted, using aseptic technique, through each IIIV guide cannula so that the distal end projected 3–5 mm past the end of the cannula and into the ventricle. The proximal end of the tubing extended ~5–10 mm above the tip of the guide cannula. Tubing was adjusted until CSF flowed easily using a blunt 22 G needle and tuberculin syringe. The tubing was then plugged until later use.

In the afternoon of the day of each experiment, ewes were assigned randomly to one of three groups ($n = 4/\text{group}$), and then ewes were placed into carts, and CSF flow was confirmed. Recombinant oleptin for use in the experiment was obtained from Ray Biotech, Inc. (Norcross, GA, USA). Treatments consisted of 1) control, Ringer–Locke buffer (pH 7.4); 2) L1, low dose of exogenous leptin (0.5 µg/kg BW); and 3) L2, high dose of roleptin (1.0 µg/kg BW) in a switchback design such that four ewes from each group received one of the three treatments (control, L1, or L2) centrally into IIIV in random order ~2 weeks apart (Fig. 1A). With 12 ewes per season, this involved three replications. The dose of leptin for i.c.v. study was determined from our previous experience (Amstalden *et al.* 2002, Zieba *et al.*

Figure 1 (A and B) Timeline for experimental procedures. 12 ewes were assigned randomly to one of three groups ($n=4/\text{group}$) during long-day (LD; March, April, May) and short-day (SD; September, October, and November) photoperiods. Treatments consisted of (1) control, Ringer-Locke buffer; (2) L1, low dose of exogenous leptin ($0.5 \mu\text{g/kg BW}$); and (3) L2, high dose of roletpin ($1.0 \mu\text{g/kg BW}$) in a switchback design such that four ewes from each group received one of the three treatments (control, L1, or L2) centrally into IIIIV in random order ~ 2 weeks apart. Arrows indicate central treatments with roletpin (L) or Ringer-Locke buffer (control, C) at 0, 1, and 2 h of the 6-h experiment.

2003a,b, 2004) and both theoretical calculations and a published study of i.c.v. injection in sheep (Adam *et al.* 2006). Blood samples (5 ml) were collected at 15-min intervals for 6 h beginning immediately before the first infusion at sunset and continued later on under the red lights (Fig. 1B).

The blood samples were dispensed into tubes containing $150 \mu\text{l}$ of a solution containing heparin ($10\,000 \text{ IU/ml}$) and 5% (w/v) EDTA and placed on ice immediately. Plasma was separated by centrifugation and stored at -20°C until melatonin, PRL, and leptin analyses.

At the end of the blood sampling experiments (June for LD season and December for SD season), a washout period of ~ 3 days followed, whereupon ewes were re-randomized and the infusion treatments described above were repeated. One hour after the end of infusions, ewes were killed humanely by captive bolt stunning. Brains were collected so that

the infundibulum remained intact. Thus, the MBH was harvested by removing a tissue block encompassing the hypothalamic–infundibular complex, followed by transection into two halves. An anterior coronal cut was made $\sim 3\text{--}5 \text{ mm}$ rostral from the optic chiasm and a posterior coronal cut was made, which included about one-third of the mamillary body. A longitudinal cut parallel to the ventral surface of the brain $\sim 2\text{--}3 \text{ cm}$ dorsal to anterior commissure followed. Tissue was frozen immediately on dry ice for storage at -70°C .

RIA

RIA for melatonin Melatonin was assayed in unextracted plasma according to the method of Fraser *et al.* (1983) and modified by Misztal *et al.* (1996). Ovine anti-melatonin serum (AB/S/01, Stockgrand Ltd, Surrey, UK), synthetic melatonin (Sigma Chemical Co.), and (*O*-methyl- ^3H)-melatonin (Amersham Biosciences) served as the first antibody, reference standard, and tracer respectively. Melatonin-free plasma for curve calibration and blanks were obtained from sheep and stripped of endogenous melatonin using activated charcoal (Norit-A; Sigma Chemical Co). The range of the calibration curve was from 15.6 to 1000 pg/ml and the working dilution of the first antibody was $1:4000$. Bound and free fractions were separated after overnight incubation at 4°C using dextran-coated charcoal. Sensitivity of the assay was $16.8 \pm 8.0 \text{ pg/ml}$ and the intra- and interassay coefficients of variations were 10.5 and 13.2% respectively.

RIA for PRL Plasma concentrations of PRL were assayed by the double antibody method using anti-ovine PRL and anti-rabbit- γ -globulin antisera according to Wolinska *et al.* (1977). Assay sensitivity was 2 ng/ml and intra- and interassay coefficients of variations were 9% and 12% respectively.

RIA for leptin Circulating concentrations of leptin were determined using a highly specific ovine leptin RIA (Delavaud *et al.* 2000). Determinations of circulating concentrations of leptin were performed in samples collected every 15 min for the first 4 h and every 30 min for the following 2 h of the experiment. The intra-assay coefficient of variation of leptin assay was 3.2% .

SOCS-3 mRNA expression in MBH Semi-quantitative expression of SOCS-3 mRNA in the MBH of the brain was performed by RT-PCR. Total RNA was prepared using TRIzol reagent (Invitrogen Corporation) according to the manufacturer's instructions. Total RNA ($1 \mu\text{g}$) was reverse transcribed to cDNA using Moloney murine leukemia virus (MMLV) Reverse Transcriptase and Oligo(dT) Primer (Advantage RT-for-PCR Kit; Clontech) using a UNO II Thermocycler (Biometra, Göttingen, Germany). An initial denaturation step was performed for 5 min at 95°C before first-strand synthesis at 42°C for 60 min . The reaction was terminated by heating to 94°C for 5 min . Each PCR

was performed by the Titanium *Taq* PCR Kit (Clontech). Primers were P1 5'-CCAGCCTGCGCCTCAA-3' and P2 5'-CTTGCGCACTGCGTTTAC-3' (corresponding to the bovine SOCS-3 gene; GenBank accession number NM_174466) for amplification of the 68 bp fragment. Primers for the reference gene were 5'-CCAACGGCTCC-CAGTTCTT-3' and 5'-ACTACGTGCTTCCCATCCA-AA-3' (corresponding to bovine cyclophilin; GenBank accession number D14074) for amplification of the 64 bp fragment. Amplification was performed by a tri-iodothyronine Thermocycler (Biometra) under the following conditions: 94 °C for 5 min followed by 36 cycles of 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 45 s. Reactions were terminated at 72 °C for 10 min. The PCR products were separated on a 3% agarose gel, stained with ethidium bromide, and analyzed using the Dscan Ex. v.3.1.0 program (Scanalytics Inc., La Jolla, CA, USA).

Statistical analysis

Hormone data were analyzed by the general linear models (GLM) procedure (PROC GLM) of the Statistical Analysis System (SAS 8.1; SAS Institute Inc., Cary, NC, USA). For hormone comparisons, the overall ANOVA included treatment, season, replicate within season, time within season, and all two and three-way interactions as for repeated measures in a switchback design. Significant treatment×season interactions resulted in a within-season model that included treatment, time and treatment×time. Following a significant *F*-test, the Pdiff procedure of SAS was used to contrast means. Content of mRNA in the MDB was evaluated by ANOVA with treatment and season as main effects. Following determination of a significant *F*-value, means were contrasted using Duncan's multiple range test. Differences with *P*<0.05 were considered statistically significant. Data are expressed as means ± S.E.M.

Results

Hormone data

In the absence of leptin treatment (control periods), mean circulating concentrations of leptin were greater (*P*<0.0001) during LD than SD (7.64 ± 0.2 vs 5.76 ± 0.1 ng/ml). Intracerebroventricular treatment with roletptin increased (*P*<0.0001) mean concentrations of circulating leptin compared with controls in both seasons in a dose-dependent manner (Fig. 2).

Mean concentrations of melatonin during control periods were greater (*P*<0.001) during LD compared with SD (61.18 ± 10.1 vs 29.66 ± 10.3 pg/ml; Fig. 3). Central roletptin infusions increased mean concentrations of melatonin (*P*<0.001) during SD in both treatment groups (L1 and L2) compared with the control (Figs 3 and 4). During LD, roletptin treatment decreased melatonin concentrations (*P*<0.001) in response to both doses of roletptin compared with the control (Figs 3 and 4).

Figure 2 Mean ($\pm$ S.E.M.) plasma concentrations of leptin in control and leptin-treated ewes during short (SD) and long days (LD). Central infusions of roletptin (L) increased (*P*<0.0001) concentrations of circulating leptin compared with control treatments in both LD and SD seasons. ***denote difference from control (*P*<0.001).

During LD, overall mean concentrations of PRL in untreated controls were much greater (*P*<0.001) than during SD (132.28 ± 19.87 vs 44.41 ± 8.27 ng/ml; Fig. 5). Intracerebroventricular infusions of both doses of roletptin decreased mean concentrations of PRL during SD (*P*<0.001, Fig. 6). Concentrations of PRL decreased in response to higher and lower dose of roletptin compared with the control in SD.

SOCS-3 gene expression in MBH

Treatment with roletptin increased (*P*<0.001) SOCS-3 mRNA expression during LD in a dose-dependent manner (Fig. 7) compared with controls. Concentrations of SOCS-3 mRNA in MBH during LD was almost twice as great (*P*<0.05) in L2 as in L1. By contrast, roletptin infusion had no effect on SOCS-3 expression during SD (Figs 7 and 8).

Figure 3 Mean ($\pm$ S.E.M.) concentrations of melatonin in control and leptin-treated ewes during short- (SD) and long-day (LD) photoperiods. Central roletptin infusions increased mean concentrations of melatonin (*P*<0.001) during SD in both treatment groups (L1 and L2) compared with controls (C). During LD, roletptin decreased melatonin concentrations (*P*<0.001) in leptin-treated sheep compared with controls in a dose-dependent manner. ***denote difference from control (*P*<0.001).

Figure 4 Temporal patterns of circulating melatonin in 15-min samples collected after control or roleptin infusions. Upper panel depicts serum concentrations of melatonin during short days (SD), with mean concentrations of melatonin greater ($P<0.001$) in L1 and L2 compared with controls (C). Lower panel depicts serum concentrations of melatonin during long days (LD) in which mean melatonin concentrations were lower ($P<0.001$) in leptin-treated ewes compared with controls. Arrows indicate Ringer-Locke buffer (control, C) or leptin treatments (L).

Discussion

The present studies confirmed our previous *in vitro* observations with pineal explants that exogenous leptin is able to differentially affect melatonin secretion in sheep depending on day length (Zieba *et al.* 2007). Herein, we report a seasonal switch in sensitivity of the ovine pineal gland to leptin, with stimulatory effects of exogenous leptin on melatonin secretion during SD and inhibitory effects during LD. Interestingly, overall mean concentrations of melatonin in control ewes were markedly greater during LD than SD. Previous reports on endogenous secretion of melatonin in sheep have indicated large inter-individual variability in plasma concentrations of melatonin at night, and this appears to be a rather common mammalian trait (Coon *et al.* 1999). Circulating concentrations of melatonin in sheep have been shown to range from less than 50 to more than 800 pg/ml, with much less variability observed within animals (Chemineau *et al.* 2002). Individual variability appears to be under strong genetic control, and originates in part from differences in pineal size and the number of pinealocytes, both of which are correlated strongly with plasma melatonin concentrations

Figure 5 Mean ($\pm$ S.E.M.) concentrations of prolactin (PRL) in control and leptin-treated ewes during short- (SD) and long-day (LD) photoperiods. Overall, mean concentrations of PRL were greater ($P<0.001$) in controls during LD compared with SD. Central infusions of roleptin (both doses) decreased ($P<0.001$) mean concentrations of PRL slightly during SD, and increased ($P<0.001$) PRL in response to two doses compared with controls during LD. ***denote difference from control ($P<0.001$).

Figure 6 Mean concentrations of circulating prolactin in 15-min samples collected after control or roleptin infusions. Upper panel depicts serum concentrations of prolactin during the short days (SD), with mean concentrations of prolactin less ($P<0.001$) in L1 and L2 treatment groups compared with controls (C). Lower panel depicts serum concentrations of prolactin during long days (LD) in which mean prolactin concentrations were increased ($P<0.001$) by roleptin compared with controls. Arrows indicate Ringer-Locke buffer (control, C) or leptin treatments (L).

Figure 7 SOCS-3 mRNA expression (% of control) in medial basal hypothalamus (MBH) of sheep during long- (LD) and short-day (SD) photoperiods. SOCS-3 expression was stimulated ($P<0.001$) by leptin during LD but not SD. * and **denote differences from controls ($P<0.05$, $P<0.001$).

(Chemineau *et al.* 2002). After collecting pineal glands from ewes for a separate *in vitro* experiment, we noted some differences in the size and weight of the pineal glands between sheep. Weight of pineal glands ranged from 60 to 150 mg (mean 87.5 mg) during LD and from 50 to 113 mg (mean 77.5 mg) during SD (Zieba *et al.* unpublished observations). Although the seasonal means in that study did not differ statistically, perhaps due to the small number of samples, it remains possible that individual variability in pineal size contributed to the major differences observed between the two seasons in the present study.

Current studies focused on the ability of leptin to differentially regulate PRL and melatonin secretion during two seasons in intact females without regard to inherent mechanisms modulating those responses, including changes in circulating ovarian steroids. While it could be argued that the mechanistic features associated with leptin-mediated changes would have been better delineated using ovariectomized, steroid-replaced ewes, a study of those features were beyond the scope of the current work. However, we do not believe that the marked differences in seasonal responses to leptin noted in the current experiments could be accounted for by the changes in circulating estradiol, progesterone, or other ovarian factors. Nonetheless, future work using ovariectomized, steroid-replaced ewes are clearly

warranted in order to determine the modulatory effects of both the estrogens and progesterone.

A circadian relationship between melatonin and leptin concentrations was reported earlier by Gündüz (2002) who suggested that photoperiod/melatonin influences the amount of circulating leptin in Siberian hamsters and that these circadian changes in leptin concentrations may be inversely linked to circulating melatonin concentrations. The mechanism of that phenomenon is not clear, but it might be due to melatonin's inhibitory effect on leptin secretion at the level of adipose cells that express melatonin receptors (Zalatan *et al.* 2001). Furthermore, receptors for melatonin have been localized in the suprachiasmatic nucleus (SCN), dorsomedial nucleus (DMN), and anterior hypothalamic area, but not in the ARC in seasonal species (Morgan & Mercer 1994). The latter would theoretically rule out the possibility of direct effects of melatonin on leptin signaling within ARC, the primary site of LRs in brain. Co-localization of receptors elsewhere has not yet been demonstrated; however, both melatonin and LRs have been independently localized in the DMN. Morgan *et al.* (2003) reported that neurons from DMN, SCN, and ARC project to the paraventricular nucleus (PVN). Adam & Mercer (2004) proposed that melatonin could contribute to hypothalamic sensitivity to leptin, acting at the region of the PVN (the center of appetite regulation), a site at which melatonin and leptin feedback may thereby be coordinated.

In another previous report in sheep, Adam *et al.* (2003) observed that a single, pharmacological i.c.v. dose of leptin specifically stimulated the frequency of luteinizing hormone (LH) pulses and simultaneously decreased appetite in the late autumn. To the contrary, no effect was observed when leptin was applied to the same sheep in the spring. However, the latest results of that group (Adam *et al.* 2006) do not support the hypothesis that leptin stimulates the reproductive neuro-endocrine axis under the influence of photoperiod; however, photoperiod modulates intrahypothalamic leptin sensitivity of appetite. These observations in sheep concerning voluntary feed intake and lack of effects on gonadotrophin-releasing hormone/LH (GnRH/LH) system are consistent with similar studies in the Siberian hamster, which are resistant to leptin during LD but become responsive to leptin treatment in terms of body weight and abdominal fat loss during SD (Atcha *et al.* 2000). Relative leptin insensitivity during LD may be necessary

Figure 8 Representative agarose gel of mRNA SOCS-3 expression in medial basal hypothalamus (MBH) of ewes during short- (SD) and long-day (LD) photoperiod (top) relative to the cyclophiline control (bottom). Lanes: M=standard weight marker; 1, 2, 3, 4=Ringer-Locke-treated sheep during LD; 5, 6, 7=leptin low dose (L1 group) LD-treated sheep; 8, 9, 10=leptin high dose (L2 group) LD-treated sheep; 11, 12, 13, 14=Ringer-Locke SD-treated sheep; 15, 16, 17=leptin low dose (L1 group) SD-treated sheep; 18, 19, 20=leptin high dose (L2 group) SD-treated sheep.

to prevent the observed increase in leptin concentrations that would cause appetite reduction and, thereby, counteract photoperiod-driven increases in voluntary food intake and body weight (Tups *et al.* 2004). Collectively, these observations imply that there is a distinct system of regulation in which normal responses to leptin and energy deficit are being overridden by photoperiod (Tups *et al.* 2004).

Our results also demonstrated a dose-related, leptin-mediated stimulation of PRL release during LD. This observation is consonant with observations of Accorsi *et al.* (2007) who noted a leptin-mediated enhancement of PRL release in bovine adenohypophyseal explants in culture, although Nonaka *et al.* (2007) reported little effect of leptin on PRL release from primary cultures of porcine anterior pituitary cells. Interestingly, Tipsmark *et al.* (2008) have shown that leptin is a very potent PRL secretagogue in teleost pituitary cultures. Secretion of PRL is tightly connected to melatonin release by the pineal gland, which is a key step in integrating the annual change in day length. In this regard, melatonin has been shown to act within the pars tuberalis (Lincoln & Clarke 1994). Melatonin receptors are highly expressed at this site, which causes the release of tuberalin, a PRL-releasing factor that regulates the synthesis and release of PRL by lactotrophs in the adjacent pars distalis (Morgan 2000). Lincoln *et al.* (2006) have proposed an intrapituitary communication between the pars tuberalis and the lactotrophs of the pars tuberalis in the regulation of the circannual PRL rhythm. The variation in plasma PRL concentrations throughout the year could also be involved in the photoperiod/melatonin effect on leptin secretion. However, it is not clear whether PRL is directly involved in adipose tissue responses to day length in sheep. Receptors for PRL on adipose tissue have been reported in mice and rats (Ling & Billing 2001, Brandebourg *et al.* 2007), and indirect effects could be mediated via secretion of a putative hepatic factor such as synlactin (English *et al.* 1990). Melatonin has also been shown to act in discrete hypothalamic areas; however, very little is known about the type of cells that are melatonin targets (Adam & Mercer 2004). That a positive relationship exists in mammals between photoperiod and PRL secretion has been known for many years, including both sheep and cattle (Tucker & Merkel 1987, Lincoln 1990). Based on the foregoing, it is possible that central infusion of leptin in the current study may have resulted in PRL secretion either by acting directly at the pituitary or through its central modulation of melatonin secretion.

In the present study, we also conducted a preliminary examination of SOCS-3 mRNA expression in the MBH, a region that includes the ARC and is assumed to play an important role in transduction of the leptin signal into a neuronal response (Zieba *et al.* 2005). The current study revealed differential SOCS-3 expression relative to SD and LD. The importance of photoperiod and melatonin in the regulation of hypothalamic leptin sensitivity in seasonal animals was demonstrated by Rousseau *et al.* (2002). In that study, hypothalamic responses to leptin in hamsters were induced primarily by photoperiod. Disinhibition of leptin signaling, represented by lower SOCS-3 mRNA expression during SD,

has been implicated in the anorectic action of leptin in that season (Klingenspor *et al.* 2000). In the current study, we observed a dose-dependent increase in the expression of the SOCS-3 gene in MBH during LD. The relationship of leptin sensitivity to SOCS-3 expression in MBH has been studied most extensively in seasonal animals such as the hamster (*Phodopus sungorus*; Klingenspor *et al.* 2000, Mercer *et al.* 2000). Data of Tups *et al.* (2006) provided evidence for SOCS-3, not only as a modulator of seasonal changes in leptin signaling, but also as a critical molecular factor in driving and timing the programmed seasonal body weight cycle. Seasonal alternations in SOCS-3 gene expression are independent of reproductive background and are primarily induced by photoperiod (Tups *et al.* 2006).

Suppressor of cytokine signaling-3 gene expression can also be induced by other photoperiodic hormones that activate signaling pathways through intracellular SOCS-3. PRL, a class I cytokine, whose action can be initiated by hormone-induced receptor homodimerization, activates receptor associated Janus kinase 2 (JAK-2) (Ling & Billing 2001). This provides a binding site for SOCS-3, which is subsequently phosphorylated by JAK-2. Activated SOCS-3 molecules then dimerize and translocate to the nucleus where they regulate the transcription of several genes and act in a feedback loop to inhibit the action of PRL (Ling & Billing 2001). However, further work would be required to clarify the possibility of photoperiodic-dependent SOCS-3 activation via PRL.

Taken together, our experiments have provided *in vivo* evidence that, in seasonal breeding sheep, responses to leptin are associated with photoperiod-driven changes in intrahypothalamic expression of mRNA SOCS-3 in the MBH. These changes were associated with leptin-mediated actions that resulted in differential regulation of both melatonin and PRL secretion during LD and SD. However, additional studies are required to determine whether the changes observed for SOCS-3 signaling are directly related to the control of melatonin or PRL secretion.

Declaration of Interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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