Hypothalamic responses to peripheral glucose infusion in food-restricted sheep are influenced by photoperiod

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Abstract

Nutritional feedback provided by systemic hormones, such as insulin and leptin, influences reproductive neuroendocrine output within the hypothalamus, yet the mechanisms and their interaction with photoperiodic cues remain unresolved in seasonal species. Here, peripheral glucose (G) infusion was used to increase endogenous concentrations of insulin and leptin in food-restricted sheep kept in either long-day (LD) or short-day (SD) photoperiod, and responses were examined in terms of pulsatile luteinising hormone (LH) (gonadotrophin-releasing hormone by inference) output and hypothalamic gene expression for nutritionally sensitive neuropeptides and receptors. We addressed the hypothesis that these hypothalamic responses were correlated and influenced by photoperiod. Oestradiol-implanted, castrated male sheep were kept 16 weeks in SD (8 h light/day) or LD (16 h light/day) and then transferred to the opposite photoperiods for 8 weeks, during which food was restricted to 90% requirement to maintain body weight (maintenance). For the final 6 days, food was reduced to 75%

maintenance, and sheep in both photoperiods were infused intravenously with G (60 mM/h) or saline (S) ($n=8$ /group). G-infused sheep had higher mean plasma concentrations of G, insulin and leptin than S-infused sheep, with no effect of photoperiod. In LD, but not in SD, G infusion increased LH pulse frequency and pulse amplitude. In LD, but not in SD, gene expression in the hypothalamic arcuate nucleus was lower in G- than S-infused sheep for neuropeptide Y (NPY) and agouti-related peptide (AGRP) and was higher in G- than S-infused sheep for pro-opiomelanocortin (POMC). Gene expression for leptin and insulin receptors was not affected by photoperiod or infusion. These results are consistent with the involvement of NPY, AGRP and POMC in mediating the reproductive neuroendocrine response to increased systemic nutritional feedback, and they support the hypothesis that hypothalamic responses to nutritional feedback are influenced by photoperiod in sheep.

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Introduction

Nutritional status influences the activity of the reproductive neuroendocrine axis through the actions of systemic nutritional feedback on gonadotrophin-releasing hormone (GnRH) neurons in the hypothalamus (I'Anson *et al.* 1991). These changes are manifested through the pulsatile secretion of luteinising hormone (LH) from the pituitary gland, which is readily measured in the circulation as a biomarker for GnRH in sheep (Clarke & Cummins 1982). The nutritional feedback may be provided in part by the metabolic hormones insulin and leptin, concentrations of which change in response to nutritional change, both in plasma (Marie *et al.* 2001) and in cerebrospinal fluid (CSF) within the brain (Miller *et al.* 1998, Blache *et al.* 2000). Furthermore, centrally administered

(intracerebroventricular (i.c.v.)) insulin and leptin have both been reported to stimulate GnRH/LH secretion in sheep (Miller *et al.* 1995, 2002, Henry *et al.* 2001a). Here, we raised endogenous concentrations of systemic insulin and leptin in sheep, simulating improved nutritional status, by peripheral glucose (G) infusion (Munoz-Gutierrez *et al.* 2002, Kadokawa *et al.* 2003). In order to maximise the potential response to the infusion, the present sheep were food restricted and therefore in negative energy balance.

In seasonal species such as sheep, however, GnRH output is primarily under photoperiodic regulation, with short days being stimulatory and long days inhibitory (Lincoln & Short 1980). The interaction and hierarchy between photoperiodic cues and nutritional feedback has yet to be resolved, but it is likely to occur within the hypothalamus. Indeed, seasonal differences in sensitivity to

leptin administered directly into the hypothalamic third ventricle have been reported for sheep (Clarke *et al.* 2001, Miller *et al.* 2002). For example, i.c.v. leptin administration suppressed appetite in autumn, but not in spring, and stimulated GnRH/LH to a greater extent in spring than in autumn, in the study of Miller *et al.* (2002). The mechanisms underlying responses to improved nutritional feedback in the present study were therefore examined in both long- and short-day photoperiods.

Seasonal changes in GnRH/LH secretion in sheep are associated with seasonal changes in concentrations of and sensitivity to circulating gonadal steroids (Legan & Karsch 1980). In addition, nutritional stimulation of GnRH/LH seems to require the presence of gonadal steroids, with responses being observed in entire rams (Martin & Walkden-Brown 1995), but not in castrated rams (Tjondronegoro *et al.* 1996), and in ovariectomised ewes only after oestradiol replacement (Rhind *et al.* 1989, 1991). This study therefore used steroid-implanted castrated males to compare infusion and photoperiod treatments between animals with a standardised, constant physiological level of gonadal steroid feedback to the hypothalamus. Sheep of the Scottish Blackface breed were used since they exhibit both distinctive seasonal reproductive cycles (Lincoln *et al.* 1990) and clear reproductive neuroendocrine responses to nutritional manipulations (Rhind *et al.* 1989, Archer *et al.* 2002).

Modulation of GnRH by insulin or leptin is likely to involve interneurons, since GnRH neurons do not express the leptin receptor (OB-Rb) (monkeys: Finn *et al.* 1998; rats: Hakansson *et al.* 1998), and there are no reports of GnRH cells expressing the insulin receptor (IR). Candidate nutritionally sensitive interneurons are those primarily implicated in appetite and energy balance, such as the orexigenic neuropeptide Y (NPY) (Kalra & Kalra 1996) and the anorexigenic melanocortin pathways (reviewed by Schioth & Watanobe 2002). Synaptic contacts with GnRH neurons have been demonstrated for NPY (in sheep: Tillet *et al.* 1989) and pro-opiomelanocortin (POMC) neurons (rat: Leranth *et al.* 1988), and i.c.v. NPY administration decreases GnRH/LH output in sheep (McShane *et al.* 1992). Melanocortin agonists and antagonists can also affect GnRH and LH release in rodent models *in vitro* and *in vivo* (Stanley *et al.* 1999, 2003). Food restriction in sheep increases hypothalamic gene expression for NPY and agouti-related peptide (AGRP) and decreases gene expression for POMC (McShane *et al.* 1993, Adam *et al.* 1997, Henry *et al.* 2001b, Archer *et al.* 2004). Centrally administered i.c.v. leptin reverses these changes in gene expression in sheep (Henry *et al.* 1999), and i.c.v. insulin downregulates NPY gene expression in rats (Schwartz *et al.* 1992). Thus, it was anticipated that the neuropeptide responses to the increased systemic nutritional feedback in the present study would be maximised by using food-restricted sheep.

The objective of this study was to determine in long days and short days the effects of raising systemic concentrations

of insulin and leptin in sheep by peripheral G infusion in terms of GnRH/LH secretion and hypothalamic gene expression for insulin and leptin receptors, NPY and components of the melanocortin pathway. We tested the hypothesis that the responses by these candidate hypothalamic neuropeptide pathways correlate with the GnRH/LH responses and that both are influenced by photoperiod.

Materials and Methods

Animals and treatments

All procedures were licensed under the Animals (Scientific Procedures) Act of 1986 and received prior approval from the Macaulay Institute's ethical review committee.

Adult castrated male Scottish Blackface sheep ($n=32$) with initial body weight of 39.9 ± 0.67 kg and body condition score (BCS) of 2.1 ± 0.02 (Russel *et al.* 1969) were group housed in either short-day (SD; 8 h light:16 h darkness) or long-day artificial photoperiods (LD; 16 h light:8 h darkness) for 16 weeks ($n=16$ /group), given daily 0.7 kg/head complete diet (83% DM; 11.6 MJ ME/kg/DM; 'Soay mix', North Eastern Farmers, Turriff, UK) and allowed hay *ad libitum*. At week 6, each sheep was given two subcutaneous, oestradiol-containing implants (Adam & Findlay 1998), which produced constant mean plasma oestradiol concentrations of 3.2 ± 0.15 pg/ml (by radioimmunoassay (RIA) of Mann *et al.* 1995). The photoperiods were then reversed for 8 weeks. The sheep were kept in individual pens and given the complete diet daily at 1000 h in amounts calculated for each individual to provide 90% of requirements for maintenance of body weight ('maintenance' = 0.42 MJ/kg^{0.75}) (Robinson 1983). During week 6, the sheep were transferred into metabolism crates, and the daily ration was reduced to 75% maintenance for the following 2 weeks. The infusion was conducted during the final 6 days of this 2-week period. Mean body weight and BCS at the start of infusion were 38.6 ± 0.76 kg and 2.0 ± 0.02 respectively. Sheep in each photoperiod were intravenously (i.v.) infused, via indwelling jugular catheters linked to a remote peristaltic pump, with either G (40% w/v; Arnolds Veterinary Products, Shropshire, UK) at a rate of 60 mM/h (27 ml/h) or saline (S) at 27 ml/h (Aquapharm, sodium chloride 0.9%; Animal Care Ltd, York, UK) ($n=8$ /treatment). This infusion protocol for sheep has been reported to induce a fourfold increase in plasma G and an 18-fold increase in plasma insulin concentrations by day 4 (Downing *et al.* 1995).

Measurements and tissue collection

Blood samples were collected by jugular venepuncture before feeding at the start, middle and end of the infusion period for metabolic analyses. On the day before the start

of i.v. infusion, serial blood samples (2.5 ml) were taken for LH analysis via jugular catheters every 15 min for 8 h, starting at 0930 h. This was repeated on day 6 of the infusion period from 0000 h to 0800 h. Plasma was stored at -20°C until assayed. For reasons of welfare and to confirm the efficacy of the infusion protocol, daily blood samples were monitored instantly for plasma G concentrations with a Glucotrend meter (Boehringer Mannheim UK Ltd, Lewes, UK). At the end of infusion, the sheep were killed by lethal i.v. injection of sodium pentobarbitone (Euthesate, Rhone Merieux Ltd, Harlow, UK), in turn from each group between 0900 and 1500 h. CSF was sampled from the cisterna magna via a spinal needle inserted between the occiput and the first cervical vertebra, and stored at -20°C ; the brain was excised, snap-frozen in isopentane over dry ice and stored at -80°C .

Hormone and metabolite analyses

Plasma leptin concentrations were determined by RIA (Marie *et al.* 2001) with a sensitivity of 0.5 ng/ml and intra- and interassay CV of 12.0% and 16.0% respectively. Insulin was measured by RIA (MacRae *et al.* 1991) with assay sensitivity of 0.2 $\mu\text{IU/ml}$, and intra- and interassay CV of 5.1% and 7.0% respectively. LH was measured by RIA (McNeilly *et al.* 1986) with a sensitivity of 0.2 ng/ml and intra- and interassay CV of 7.0% and 10% respectively. Non-esterified fatty acids (NEFA) and G concentrations were determined by automated KONE analyser, with sensitivities of 0.04 and 0.34 mmol/l respectively.

Hypothalamic gene expression

Coronal cryostat hypothalamic sections (20 μm) were thaw-mounted onto slides double-coated with gelatin and poly-L-lysine, and stored at -80°C . Gene expression for NPY, AGRP, POMC, OB-Rb and IR was measured by *in situ* hybridisation. Corresponding sense and antisense probes were generated. The NPY riboprobe was generated from a rat cDNA and has been validated in sheep brain (Adam *et al.* 1997). The AGRP and POMC probes were generated from cloned Siberian hamster cDNAs (Mercer *et al.* 2000) and have been validated in sheep brain (Adam *et al.* 2002). A riboprobe complementary to fragments of the intracellular domain of OB-Rb was generated from a cloned sheep cDNA, as described previously (Mercer *et al.* 1998). The riboprobe for IR was generated from a 726 bp partial ovine IR sequence corresponding to nucleotides 203–933 of the human insulin receptor open reading frame (Accession no. NM_000208), which had been generated by PCR of ovine adipose tissue cDNA, with primers 5'-CCC GAAGATTTCCGAGACCTCAGTTTCCC-3' and 5'-GCACTTGTTGTTGTG TGAATGACGTATTGGTG-3', and cloned into the pGEM-7zf+ vector. Use of this IR riboprobe has not been

reported for *in situ* hybridisation in sheep brains before. The nucleotide sequence of the ovine insulin receptor cDNA will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number AJ844652.

The *in situ* hybridisation technique has been described in detail elsewhere (Mercer *et al.* 1995, Adam *et al.* 1997). Briefly, sections were fixed, acetylated and hybridised overnight at 58°C with ^{35}S -labelled cRNA probes ($1\text{--}1.5 \times 10^7$ c.p.m./ml). They were then treated with RNase A, desalted, with a final high stringency wash (30 min) in $0.5 \times \text{SSC}$ at 60°C (IR at 75°C), dried and apposed to Hyperfilm β -max (Amersham Pharmacia Biotech UK Ltd, Little Chalfont, UK) for 7 days. Intensity of hybridisation was quantified on autoradiographic images, using Image-Pro Plus (Media Cybernetics, Silver Spring, MD, USA). The integrated intensity of the hybridisation signal was computed by standard curves generated from ^{14}C autoradiographic microscaler (Amersham Pharmacia Biotech). For each probe, three sections were analysed in each brain from each of these three hypothalamic regions: caudal hypothalamus (just anterior to the mammillary body, where the third ventricle extends to the base of the brain), medial hypothalamus (approximately 1.5 mm anterior to caudal sections) and rostral hypothalamus (approximately 2.5 mm anterior to caudal sections). In addition, for the IR riboprobe, three sections were studied through the paraventricular nucleus (PVN) (approximately 3.0 mm anterior to the caudal sections, where the optic chiasm extends across the ventral margin of the hypothalamus). All sections for a single probe were processed together and placed against the same sheet of autoradiographic film.

All reagents were obtained from Sigma unless otherwise stated.

Statistical analysis

Serial LH data were analysed with a version of the 'Pulsar' algorithm (Merriam & Wachter 1982) modified for the Apple Macintosh computer ('Munro', Zaristow Software, West Morham, Haddington, East Lothian, UK). LH pulse frequency, pulse amplitude, baseline and mean concentrations were obtained for each individual's profiles before and after the infusion period. Significance of changes in LH pulse frequency and mean LH were then determined by Wilcoxon matched-pairs signed-rank tests and paired *t*-tests respectively, and that of changes in pulse amplitude and baseline concentrations by sign tests. Two-way ANOVA was used to compare treatment groups with respect to plasma concentrations of insulin, leptin, G and NEFA, CSF concentrations of G and insulin, and arcuate nucleus (ARC) gene expression data. All statistical tests were performed with Genstat 5 (Release 4.1, Lawes Agricultural Trust, IACR-Rothamsted, Harpenden, UK).

Figure 1 Plasma concentrations of (a) glucose, (b) insulin, (c) leptin and (d) NEFA before (open bars) and after (solid bars) 6-day peripheral infusion with saline (S) or glucose (G) in food-restricted sheep kept for 8 weeks in long-day (LD) or short-day (SD) photoperiod. ** $P < 0.01$, *** $P < 0.001$.

Results

Metabolic status

Body-weight loss during the infusion period was not different between the groups, averaging 2.0 ± 0.22 kg for all sheep. Plasma G concentrations were similar in SD and LD sheep at the start of infusion and remained similar between SD and LD on day 6 of infusion, but were approximately fourfold higher in G than saline (S) treatments ($P < 0.001$) (Fig. 1). Plasma insulin concentrations were similar between SD and LD, but after the 6 days of infusion they were approximately fivefold higher in G than S treatments in both photoperiods ($P < 0.001$) (Fig. 1). Plasma leptin concentrations were higher in SD than LD ($P < 0.01$), but were 1.7 times higher in G than S treatment groups in both photoperiods ($P < 0.001$) (Fig. 1). Plasma NEFA concentrations were lower in SD than LD ($P < 0.01$) and were reduced approximately eightfold by G compared with S treatment in both photoperiods ($P < 0.001$) (Fig. 1).

Mean concentrations of G and insulin in CSF were similar in SD and LD animals, but were significantly higher in G- than S-infused sheep ($P < 0.001$) (Fig. 2).

Concentrations of leptin were below the detection limit of the assay in all CSF samples.

LH secretion

At the start of infusion, LH secretory parameters were not different between the photoperiods. On day 6 of infusion, G treatment in LD had increased LH pulse frequency ($P < 0.05$) and pulse amplitude ($P < 0.01$), but not in SD (Fig. 3). The increase in mean LH concentration during G infusion in LD failed to reach significance ($P = 0.07$). By contrast, S treatment had no significant effect on these LH secretory parameters. Baseline LH concentrations were unaffected by photoperiod or infusion (Fig. 3).

Hypothalamic gene expression

Since the corresponding sense probes showed no hybridisation, the hybridisation of antisense probes to the ovine hypothalamic sections indicated the presence of the specific mRNAs.

NPY, AGRP and POMC mRNAs were detected and quantified in the caudal, medial and rostral regions

Figure 2 Cerebrospinal fluid (CSF) concentrations of (a) glucose and (b) insulin after 6-day peripheral infusion with saline (S; dotted bars) or glucose (G; striped bars) in food-restricted sheep kept for 8 weeks in long-day (LD) or short-day (SD) photoperiod. *** $P < 0.001$.

of the ARC. Since treatment differences were similar across the regions, overall mean values for the whole ARC were used in the final statistical analysis for clarity of presentation. Amounts of AGRP and POMC mRNAs were not affected by photoperiod alone, but the amount of NPY mRNA was greater in S-infused sheep in LD than in SD. Mean amounts of NPY and AGRP mRNAs were lower in G than S treatment groups in LD ($P < 0.05$ and $P < 0.01$ respectively), but not in SD (Figs 4 and 6). POMC gene expression was higher in G than S treatments in LD ($P < 0.01$), but not in SD (Figs 4 and 6).

OB-Rb gene expression levels in the ARC were similar across caudal, medial and rostral regions and were not affected by photoperiod or infusion treatment (Fig. 6). Gene expression for IR was detected for the first time in ovine hypothalamus, with localisation concentrated in the medial ARC and the PVN, as well as in the pars tuberalis (PT) of the pituitary (Fig. 5). Amounts of IR mRNA in the ARC and PVN were not significantly affected by photoperiod or infusion treatment (Fig. 6).

Discussion

The G infusion protocol increased circulating concentrations of insulin and leptin in the sheep in both photoperiods. However, only in LD was GnRH/LH output stimulated, NPY and AGRP mRNAs downregulated, and POMC mRNA upregulated. The results therefore support the hypothesis that photoperiod influences the hypothalamic responses to increased insulin and/or leptin feedback. Furthermore, the data are consistent with the putative involvement of NPY and melanocortin pathways in mediating the GnRH/LH response to improved nutritional feedback.

The fourfold increase in plasma G increased circulating concentrations of insulin (5-fold) and leptin (1.7-fold), the latter being induced either directly or indirectly, as through insulin stimulation (Saladin *et al.* 1995, Pagano *et al.* 1997). The increase in LH output (and GnRH by inference) (Clarke & Cummins 1982) seen in LD could have been caused by the elevated G, insulin or leptin, or a combination. Evidence for G modulation of GnRH/LH in sheep comes largely from studies in which GnRH/LH secretion is inhibited by glucoprivation, indicating a link between G-sensing mechanisms in the brain and GnRH neurons (Bucholtz *et al.* 1996, Ohkura *et al.* 2000). However, there is no evidence that G administration to euglycaemic sheep stimulates GnRH/LH. By contrast, there is good evidence for GnRH/LH stimulation following insulin or leptin administration in sheep (Miller *et al.* 1995, Daniel *et al.* 2000, Henry *et al.* 2001a, Miller *et al.* 2002). The intriguing question arising from the present study is why GnRH/LH was stimulated only in LD, despite similar elevations in plasma concentrations of insulin and leptin in both photoperiods. We have previously observed increased GnRH/LH responses to i.c.v. leptin in spring (LD) as opposed to autumn (SD) (Miller *et al.* 2002), and others have reported that photoperiod influences sensitivity to exogenous leptin in terms of body-weight change in Siberian hamsters (Atcha *et al.* 2000, Klingenspor *et al.* 2000). In agreement with seasonal changes observed in the GnRH/LH sensitivity to dietary nutritional stimulation in rams (Hotzel *et al.* 2003), it appears that LD primes the GnRH system to respond to nutritional stimulation, when photoperiod is not stimulatory, whereas photoperiodic stimulation predominates over nutritional feedback in SD.

An additional explanation for the contrasting GnRH/LH responses to G between the photoperiods may arise from the food restriction protocol used in this experiment. NEFA concentrations provide a measure of fat mobilisation, and these were decreased in response to G infusion in both photoperiods, reflecting an amelioration of the state of negative energy balance induced by the food restriction. However, although the sheep were of similar adiposity and the restricted quantity of food consumed was similar in both photoperiods, NEFA values were higher in LD than

Figure 3 (a) Luteinising hormone (LH) pulse frequency, (b) LH pulse amplitude, (c) baseline LH concentration and (d) mean LH concentration before (open bars) and after (solid bars) 6-day peripheral infusion with saline (S) or glucose (G) in food-restricted sheep kept for 8 weeks in long-day (LD) or short-day (SD) photoperiod. * $P < 0.05$, ** $P < 0.01$.

in SD, implying a functionally greater restriction. This interpretation is perhaps supported, first, by the corresponding leptin concentrations being lower in LD than SD, since food restriction decreases plasma leptin (Blache *et al.* 2000, Marie *et al.* 2001), and, second, by the increased NPY gene expression, characteristic of negative energy balance (McShane *et al.* 1993, Adam *et al.* 1997), seen in LD compared with SD for the S-infused sheep. Appetite and voluntary (*ad libitum*) food intake are normally higher in LD than SD (Adam 2000, Clarke *et al.* 2003); the intake deficit was therefore greater for the present sheep in LD than in SD, and this may have led to increased sensitivity to the increased nutritional feedback.

LH output is higher in SD (breeding season) than in LD (non-breeding season) for *ad libitum* feeding sheep (Lincoln & Short 1980). The lack of significant difference in LH secretion between the photoperiods at the start of infusion in the present experiment was largely attributable to the food restriction protocol, since undernutrition and poor nutritional feedback characteristically inhibit GnRH/LH output (McShane *et al.* 1993, Adam *et al.* 1997), and this may have prevented any photoperiodic (SD) stimulation of GnRH/LH. Alternatively, undernutrition may have

diminished the difference between the photoperiods by altering the secretory pattern of melatonin, the humoral mediator of photoperiodic feedback (Morgan & Mercer 1994), resulting in comparable levels of GnRH/LH stimulation in SD and LD. It is open to speculation whether the photoperiod-dependent increased stimulation of GnRH/LH as a consequence of the G infusion was also mediated through altered melatonin secretion. Although melatonin secretion is nutritionally sensitive in rats (Chik *et al.* 1987), with potentiation of melatonin action during food restriction (Wilamowska *et al.* 1992), there are no comparable data for seasonal species such as sheep. The stimulation of GnRH/LH as a consequence of the G infusion and positive nutritional feedback could have been partly mediated by a decrease in sensitivity to oestradiol negative feedback, since increased sensitivity during food restriction has been reported in sheep (Beckett *et al.* 1997). Furthermore, changes in sensitivity to oestradiol-negative feedback could underlie the photoperiodic differences in response to G, since sensitivity is higher in LD than in SD (Legan & Karsch 1980). It is tempting to speculate that interactive influences of photoperiod and food restriction may have facilitated the reversal of negative feedback by G infusion in LD, but not in SD.

Figure 5 Representative autoradiographic images showing hybridisation in the ovine hypothalamus to radiolabelled insulin receptor (IR) antisense (a and b) and sense (c and d) riboprobes. ARC: arcuate nucleus; PVN: paraventricular nucleus; 3 V: third ventricle; PT: pars tuberalis of the pituitary. Bar=3.1 mm.

In terms of hypothalamic gene expression, a pattern of response to G infusion emerges in the present study that is similar to that shown by the GnRH/LH axis. NPY, AGRP and POMC mRNAs were all regulated, but only in LD, consistent with their putative involvement in mediating the GnRH response. The lower gene expression of NPY and AGRP and the higher level of expression of POMC mRNA reflected a reduction in orexigenic drive brought about by the improved nutritional feedback provided by the G infusion and the increased insulin and leptin concentrations. However, this response was not elicited in SD. Photoperiod alone in *ad libitum* feeding, steroid-implanted, castrated sheep appears to have no effect on expression of these mRNAs, but NPY and AGRP mRNAs are upregulated by poor nutritional feedback during food restriction (Archer *et al.* 2004). The present data suggest that photoperiod primes the sensitivity of NPY, AGRP and POMC neurons in the ARC to improved nutritional feedback to be higher in LD and lower in SD.

There is good evidence that NPY gene expression is downregulated by i.c.v. insulin (Schwartz *et al.* 1992) and i.c.v. leptin (Henry *et al.* 1999). From rodent studies it is

Figure 4 Representative autoradiographic images showing hypothalamic sections hybridised to radiolabelled riboprobes for (a–d) NPY, (e–h) AGRP and (i–l) POMC mRNAs. Sections are from food-restricted sheep kept for 8 weeks in long-day (LD; a, b, e, f, i and j) or short-day (SD; c, d, g, h, k and l) photoperiod and given 6-day peripheral infusion with saline (S; a, c, e, g, i and k) or glucose (G; b, d, f, h, j and l). ARC: arcuate nucleus; 3 V: third ventricle. Bar=2mm.

Figure 6 Hypothalamic gene expression in the arcuate nucleus for (a) NPY, (b) AGRP, (c) POMC, (d) OB-Rb and (e) IR, and (f) in the paraventricular nucleus for IR, in food-restricted sheep kept for 8 weeks in long-day (LD) or short-day (SD) photoperiod and given 6-day peripheral infusion with saline (S; dotted bars) or glucose (G; striped bars). ** $P<0.01$, * $P<0.05$.

also known that gene expression of AGRP, the endogenous antagonist to the melanocortin pathway that coexpresses with NPY (Mercer *et al.* 2000), is also downregulated by leptin (Wilson *et al.* 1999). Conversely, gene expression of the melanocortin precursor POMC is upregulated by insulin (Kim *et al.* 1999, Benoit *et al.* 2002), and electrophysiological recordings demonstrate that leptin activates POMC neurons (Cowley *et al.* 2001). The present data are therefore consistent with the observed neuropeptide changes being mediated by the raised insulin and/or leptin signal. There is little corresponding evidence for direct effects of G on expression of

these genes. The altered sensitivity of the NPY, AGRP and POMC neurons between the photoperiods was not associated with altered receptor gene expression for insulin or leptin in the ARC and was therefore more likely to have been mediated by changes within the cell. Indeed, recent evidence from another seasonal animal, the Siberian hamster, suggests that photoperiod can regulate expression of SOCS-3, a critical player in intracellular leptin signalling (Tups *et al.* 2004).

IR gene expression measured by *in situ* hybridisation in the ovine hypothalamus has not previously been reported. In addition to extrahypothalamic localization in the PT, it

was localised in the ARC, consistent with findings in the rat hypothalamus (Marks *et al.* 1990) and also in the PVN, a finding not previously reported. IR gene expression was not affected at either site by G infusion, despite the increased circulating and CSF insulin concentrations; this is consistent with the lack of regulation by insulin reported for central IR in rats (Schwartz *et al.* 1992). Leptin receptor gene expression in the ARC was also not affected by G infusion. This seemingly contrasts with the reported downregulation of OB-Rb mRNA by i.c.v. insulin in the undernourished sheep of Daniel *et al.* (2000). The discrepancy may be due to the fact that their animals were ovariectomised, without steroid replacement, and they had both a more severe level of food restriction and a longer period of insulin infusion. Interestingly, again in contrast to our present data, i.c.v. insulin did not affect NPY mRNA expression in the sheep of Daniel *et al.* (2000). Perhaps surprisingly, OB-Rb gene expression in the present experiment was apparently unaffected by the increase in circulating concentrations of its ligand. However, although a decrease in leptin due to food restriction or deprivation almost invariably upregulates OB-Rb, there are fewer reports of the effects of hyperleptinaemia on OB-Rb expression (Ahima & Flier 2000); furthermore, photoperiod cues seem to desensitise the hypothalamic leptin receptor response to changes in leptin concentrations in seasonal mammals (Mercer *et al.* 2001, Archer *et al.* 2004).

Finally, the present data are consistent with mediation of the observed GnRH/LH response by NPY and/or melanocortin pathways. It is postulated that NPY mRNA expression, raised by the food restriction, was inhibiting GnRH/LH output (Malven *et al.* 1992, McShane *et al.* 1993, Adam *et al.* 1997); the increased circulating G, insulin and leptin would have then reduced the NPY mRNA expression, thereby stimulating or releasing the inhibition of GnRH (McShane *et al.* 1992). In addition, rodent studies indicate that there are synaptic contacts between POMC and GnRH neurons (Leranth *et al.* 1988), and the POMC product α -MSH is thought to mediate leptin stimulation of GnRH (Watanobe 2002). Thus, the raised POMC gene expression in the present experiment could have led to GnRH/LH stimulation by this route; in addition, the reduced AGRP gene expression would have reduced antagonism at the melanocortin receptors (Ollmann *et al.* 1997) and could have contributed to the net stimulation of GnRH by increased melanocortin activity.

Therefore, these results are consistent with an increase in hypothalamic responsiveness to improved peripheral nutritional feedback during food restriction in LD compared with SD; and this was evident in both appetite-regulatory pathways and the reproductive neuroendocrine axis. Peripheral G infusion in food-restricted sheep, with resulting increases in circulating insulin and leptin, influenced both hypothalamic NPY, AGRP and POMC gene

expression and GnRH/LH pulsatile secretion in a photoperiod-dependent fashion, consistent with mediation of the GnRH/LH response by NPY and melanocortin pathways.

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