Contrasting effects of different levels of food intake and adiposity on LH secretion and hypothalamic gene expression in sheep

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Abstract

Body reserves (long-term) and food intake (short-term) both contribute nutritional feedback to the hypothalamus. Reproductive neuroendocrine output (GnRH/LH) is stimulated by increased food intake and not by high adiposity in sheep, but it is unknown whether appetite-regulating hypothalamic neurons show this differential response. Castrated male sheep (Scottish Blackface) with oestradiol implants were studied in two 4 week experiments. In Experiment 1, sheep were fed to maintain the initial body condition (BC) score of 2.0 ± 0.00 (lower BC (LBC), $n=7$) or 2.9 ± 0.09 (higher BC (HBC), $n=9$), and liveweight of 43 ± 1.1 and 59 ± 1.6 kg respectively. LBC and HBC sheep had similar mean plasma LH concentration, pulse frequency and amplitude, but HBC animals had higher mean plasma concentrations of insulin ($P<0.01$), leptin ($P<0.01$) and glucose ($P<0.01$). Gene expression (measured by *in situ* hybridisation) in the hypothalamic arcuate nucleus (ARC) was higher in LBC than HBC sheep for neuropeptide Y (NPY; 486% of HBC, $P<0.01$), agouti-related peptide (AGRP; 467%, $P<0.05$) and leptin receptor (OB-Rb; 141%, $P<0.05$), but lower for cocaine- and amphetamine-regulated transcript (CART; 92%, $P<0.05$) and similar between groups for pro-opiomelanocortin (POMC). In Experiment 2, sheep with initial mean BC score 2.4 ± 0.03 and liveweight 55 ± 0.8 kg were fed a liveweight-maintenance ration

(low intake, LI, $n=7$) while sheep with initial mean BC score 2.0 ± 0.03 and liveweight 43 ± 1.4 kg were fed freely so that BC score increased to 2.5 ± 0.00 and liveweight increased to 54 ± 1.4 kg (high intake, HI, $n=9$). Compared with LI, HI sheep had higher mean plasma LH ($P<0.05$), baseline LH ($P<0.01$) and pulse amplitude ($P<0.01$) and showed a trend towards higher pulse frequency. Although there were no differences in final mean plasma concentrations, there were significant increases over time in mean concentrations of insulin ($P<0.001$), leptin ($P<0.05$) and glucose ($P<0.001$) in HI sheep. Gene expression for AGRP in the ARC was higher in HI than LI animals (453% of LI; $P<0.05$), but expression levels were similar for NPY, OB-Rb, CART and POMC. Thus, the hypothalamus shows differential responses to steady-state adiposity as opposed to an increase in food intake, in terms of both reproductive neuroendocrine activity and hypothalamic appetite-regulating pathways. Differences in hypothalamic gene expression were largely consistent with contemporary levels of systemic leptin and insulin feedback; however, increased nutritional feedback was stimulatory to GnRH/LH whereas constant high feedback was not. The hypothalamus therefore has the ability to retain a nutritional memory that can influence subsequent responses.

Journal of Endocrinology (2002) **175**, 383–393

Introduction

Nutritional feedback from the periphery to the hypothalamus regulates the appetite/bodyweight and reproductive neuroendocrine axes, and a comprehensive knowledge of how the hypothalamus responds to this feedback is critical for the understanding and control of appetite/bodyweight disorders, such as obesity, and infertility. Nutritional feedback comprises both long-term and short-term components derived from body energy reserves and contemporary food intake respectively, yet

these components are frequently confounded in animal experiments. However, reproductive neuroendocrine output, measured by pulsatile luteinising hormone (LH) secretion, is stimulated by short-term increases in food intake ('flushing') in ewes (Rhind *et al.* 1985, 1986, 1991) and rams (Martin & Walkden-Brown 1995), but not by long-term increases in body fat reserves in ewes (Rhind & McNeilly 1986, Rhind *et al.* 1989). Similarly, LH secretion is reduced in rams on restricted feeding, but pituitary LH content is not different between maintenance-fed rams with different levels of body fat

(Alkass *et al.* 1982). Since LH secretion is an indirect measure of hypothalamic gonadotrophin-releasing hormone (GnRH) pulsatile output (Clarke & Cummins 1982), these findings suggest that the hypothalamus can dissociate long- and short-term nutritional feedback with respect to GnRH activity. It is not known how the differential GnRH response is mediated or how the orexigenic-anorexigenic hypothalamic neuronal circuitry responds to the differential nutritional feedback.

Candidate signalling mechanisms for the provision of nutritional feedback from peripheral tissues to the hypothalamus include leptin and insulin, since their secretion is influenced by both body condition (BC) and food intake in sheep (Marie *et al.* 2001). Both hormones are implicated in nutritional modulation of reproduction (Blache *et al.* 2000a) and in appetite/bodyweight regulation (Schwartz *et al.* 2000). Insulin administered in physiological amounts into the ovine hypothalamus stimulates GnRH/LH (Miller *et al.* 1995). We have repeated this effect with physiological intracerebroventricular (i.c.v.) infusion of leptin (Miller *et al.* 2002), although published responses to pharmacological i.c.v. leptin administration of GnRH/LH in sheep are variable (Henry *et al.* 1999, 2001a, Blache *et al.* 2000b, Morrison *et al.* 2001). Both hormones have also been shown to reduce appetite in sheep when given i.c.v. (Foster *et al.* 1991, Henry *et al.* 1999, 2001a, Blache *et al.* 2000b, Morrison *et al.* 2001).

Anatomical studies of gene expression have indicated that insulin receptors and leptin receptors (OB-Rb) are localised in the ovine hypothalamic arcuate nucleus (ARC) (Z A Archer, P A Findlay & C L Adam, unpublished observations, and Williams *et al.* (1999) respectively). Studies in rodents suggest that leptin and insulin exert their actions on appetite and energy balance at least in part via the orexigenic neuropeptides neuropeptide Y (NPY) and agouti-related peptide (AGRP, melanocortin antagonist), and the anorexigenic pro-opiomelanocortin (POMC, melanocortin precursor) and cocaine- and amphetamine-regulated transcript (CART) neuronal pathways (reviewed by Schwartz *et al.* 2000). Gene expression for these neuropeptides has been localised in the ovine ARC (Adam *et al.* 2002). The roles of these orexigenic-anorexigenic pathways in the actions of insulin and leptin on GnRH secretion are unresolved, but GnRH cells have close synaptic contacts with both NPY and POMC neurons in rats (Kalra & Kalra (1996) and Leranth *et al.* (1988) respectively). I.c.v. administration of NPY to sheep (Malven *et al.* 1992, McShane *et al.* 1992) and of the POMC product α -melanocyte-stimulating hormone (α MSH) to rats (Gonzalez *et al.* 1997) inhibits GnRH/LH pulsatile secretion while AGRP administered i.c.v. stimulates GnRH/LH output in rats (Stanley *et al.* 1999) and CART stimulates GnRH release from rat hypothalamic explants *in vitro* (Lebrethon *et al.* 2000).

The castrated male with oestradiol replacement is an accepted model used in studies of nutrition-reproduction neuroendocrine interactions in sheep (e.g. Adam & Findlay 1998, Nagatani *et al.* 2000) and was our model of choice for the present study. It avoids the changes in concentrations of circulating gonadal steroid seen across seasons in gonad-intact animals of both sexes and across the oestrous cycle in ewes, whilst providing a constant physiological level of gonadal steroid feedback to the hypothalamus. Furthermore, the GnRH/LH response to 'flushing' is observed in entire rams (Martin & Walkden-Brown 1995), but not in castrated rams (Tjondronegoro *et al.* 1996), and is seen in ovariectomised ewes only in the presence of oestradiol (Rhind *et al.* 1989, 1991).

In order to extend our knowledge of hypothalamic responses to different scenarios of nutritional feedback, we investigated in sheep two experimental paradigms aimed at dissociating the effects of differential adiposity (at similar food intake) and differential food intake (at similar levels of adiposity). Measurements were made of systemic leptin and insulin concentration patterns and associated GnRH/LH secretion and hypothalamic ARC gene expression for OB-Rb, NPY, AGRP, POMC and CART. A differential GnRH/LH response was expected between these paradigms and we addressed the hypothesis that orexigenic and anorexigenic hypothalamic neuronal pathways would also exhibit differential responses.

Materials and Methods

Animals and preparatory treatments

Adult castrated male Scottish Blackface sheep ($n=32$), with an initial mean liveweight 48 ± 0.9 kg and BC score 2.25 ± 0.02 (scale 0 = emaciated to 5 = obese; Russel *et al.* (1969)), were housed in individual pens under natural photoperiod (August; 57°N). Over a 9 week period prior to Experiments 1 and 2, each sheep was given 0.25 kg hay/day and an amount of pelleted concentrate feed calculated to induce the desired weight change (ranging from 0.2 to 2.5 kg/day) (Green Keil; North Eastern Farmers, Turriff, UK (86.1% dry matter (DM) and 12.5 MJ metabolisable energy (ME)/kg DM)). The amounts fed were adjusted as necessary on an individual basis with the objective of achieving four groups of sheep with mean BC scores of 2.0 and 3.0 at the beginning of Experiment 1, or 2.0 and 2.5 at the beginning of Experiment 2. No individual was required to increase or decrease more than 0.75 BC score units. Food was given once daily at 0800 h.

At 5 weeks before the beginning of the experiments two oestradiol-containing implants were inserted s.c. (Adam & Findlay 1998). Circulating oestradiol concentration, determined by RIA with sensitivity 0.42 pg/ml (Mann *et al.* 1995), averaged 2.1 ± 0.14 pg/ml (range 1.03–3.45 pg/ml).

All procedures were licensed under the UK Animals (Scientific Procedures) Act of 1986 and received prior approval from the Macaulay Land Use Research Institute's Ethical Review Committee.

Experiment 1

Mean initial BC scores were 2.0 ± 0.00 (lower BC (LBC), $n=7$) and 2.9 ± 0.09 (higher BC (HBC), $n=9$), representing estimated body fat contents of 20 and 29% respectively (Russel *et al.* 1969); and mean initial liveweights were 43 ± 1.1 and 59 ± 1.6 kg respectively. During the 4 week experimental period, the animals were fed individually a total amount calculated to maintain liveweight and BC score (0.42 MJ ME/kg^{0.75}/day; Robinson (1983)), comprising hay and pelleted feed given once daily as described above.

Experiment 2

Mean BC scores were 2.4 ± 0.03 and 2.0 ± 0.03 in the two groups at the beginning of the experimental period, representing body fat contents of 24 and 20% respectively (Russel *et al.* 1969); and mean initial liveweights were 55 ± 0.8 and 43 ± 1.4 kg respectively. Animals of the BC 2.4 group were fed amounts calculated on an individual basis, as above, to maintain liveweight and BC score for 4 weeks (low intake (LI), $n=7$). Sheep in the BC 2.0 group were fed freely for 4 weeks so that their mean BC score would increase to approximately 2.5 (high intake (HI), $n=9$). The food refusals (15% margin) were removed and recorded daily, just prior to the provision of fresh food at 0800 h, in order to calculate voluntary food intake.

Measurements and tissue collection

Liveweights and BC scores were measured weekly, and voluntary food intakes were measured daily in the HI group. Blood samples were collected twice weekly, approximately 1.5 h after feeding, and immediately prior to killing. During week 4 of the experimental period, blood samples were collected via jugular catheters every 15 min for 7.5 h ('pulse bleed'), starting at 0830 h (30 min after feeding), to determine pulsatile LH secretion. Plasma was stored at -20°C for subsequent LH analysis.

Within 1–2 days of the pulse bleed, sheep were killed by a lethal dose of sodium pentobarbitone (i.v.) (Euthesate; Willows Francis Veterinary, Crawley, Sussex, UK) between 0930 and 1630 h, following feeding as usual at 0800 h. Cerebrospinal fluid (CSF) was sampled from the cisterna magna 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 concentrations of LH were measured by RIA (McNeilly *et al.* 1986) with an assay sensitivity of

0.2 ng/ml and intra- and inter-assay coefficient of variation (CV) values of 4.4 and 12.9% respectively. Non-esterified fatty acid (NEFA) and glucose concentrations were determined by automated analysis, with sensitivity and intra- and inter-assay CV values of 0.04 mmol/l, 2.0 and 4.0% respectively for NEFA (Matsubara *et al.* 1983), and 0.34 mmol/l, 0.35 and 2.3% respectively for glucose (Peterson & Young 1968). Leptin concentrations were determined by homologous RIA (Marie *et al.* 2001) in a single assay with a sensitivity of 0.45 ng/ml and intra-assay CV of 12.0%. Insulin was measured by RIA (MacRae *et al.* 1991) in a single assay run showing sensitivity of 0.2 $\mu\text{IU/ml}$ and an intra-assay CV of 2.7%.

Hypothalamic gene expression

Coronal cryostat sections (20 μm) of hypothalamic tissue were thaw-mounted onto slides double-coated with gelatin and poly-L-lysine, and stored at -80°C . Gene expression for OB-Rb, NPY, AGRP, POMC and CART was measured by *in situ* hybridisation, using techniques described in detail elsewhere (Mercer *et al.* 1995, Adam *et al.* 1997). 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 NPY probe was generated from a rat cDNA and has been validated for use on sheep tissues (Adam *et al.* 1997). The CART probe was generated from a cloned sheep cDNA as described previously (Barrett *et al.* 2001). AGRP and POMC probes were generated from cloned Siberian hamster cDNAs (Mercer *et al.* 2000) and have been validated on sheep brain tissue (Adam *et al.* 2002). Briefly, sections were fixed, acetylated, and hybridised overnight at 58°C using ³⁵S-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 , dried and apposed to Hyperfilm $\beta\text{-max}$ (Amersham Pharmacia Biotech UK Ltd, Little Chalfont, Bucks, UK). Intensity and total area of hybridisation over the ARC were quantified on each autoradiographic image, using the Image-Pro Plus system (Media Cybernetics, Silver Spring, MD, USA). The integrated intensity of the hybridisation signal was then computed using standard curves generated from ¹⁴C autoradiographic micro-scales (Amersham Pharmacia Biotech). For each probe, three comparable sections from the medial hypothalamus (approximately 1.5–1.75 mm rostral from the opening of third ventricle) were analysed for each brain and the results averaged to give a single value for each animal. Within each experiment, all sections for a single probe were processed together and placed against the same sheet of autoradiographic film.

All reagents were obtained from Sigma (Sigma UK, Poole, Dorset, UK) unless otherwise stated.

Table 1 CSF concentrations of glucose and insulin (means ± S.E.M.) in sheep fed at liveweight maintenance with HBC or LBC (Experiment 1) and sheep with similar terminal BC scores fed freely (HI) or a liveweight-maintenance ration (LI) (Experiment 2). Leptin concentrations were below the detection limit of the assay (i.e. less than 0.45 ng/ml) in all groups. There were no significant differences

	Experiment 1		Experiment 2	
	HBC (n=9)	LBC (n=7)	LI (n=7)	HI (n=9)
Glucose (mmol/l)	2.3 ± 0.14	2.0 ± 0.19	2.2 ± 0.12	2.2 ± 0.11
Insulin (µIU/ml)	3.7 ± 0.18	3.7 ± 0.53	3.6 ± 0.20	3.9 ± 0.21

Statistical analyses

Mean ± S.E.M. values are reported and the probability value used for statistical significance was $P<0.05$. Initially, mean plasma concentrations of insulin, leptin, glucose and NEFA for the duration of the experiments were calculated within individuals and compared between treatment groups within Experiments 1 and 2 using two-sample t -tests. The trends in these parameters over time appeared relatively linear and were therefore analysed by comparing mean linear trend coefficients (regression slopes) (Diggle *et al.* 1994). Linear regression slopes were calculated for each animal for each parameter over the 4 week experimental period and confidence intervals for the regression slopes were used to assess whether values changed over time. Comparison of the linear regression slopes using two-sample t -tests was undertaken to assess the difference in the trends between groups (treatment × time interaction) within each experiment. Linear regressions of BC score and food intake against time were similarly compared between groups using two-sample t -tests.

LH profiles were analysed using PulseFit (Version 1.3; R H Kushler, Department of Mathematical Sciences, Rochester, MI, USA), which provided values for LH pulse frequency, pulse amplitude and baseline concentrations. In addition, within-animal mean concentrations were calculated for each pulse bleed profile ('mean LH'). Treatment effects on pulse amplitude, baseline LH and mean LH were analysed by one-way ANOVA and effects on LH pulse frequency by Wilcoxon Mann–Whitney tests. Levels of gene expression were compared between the groups within each experiment by one-way ANOVA. All statistical tests were carried out using Microsoft Excel 97 (Microsoft, Redmond, WA, USA).

Results

Liveweight, BC and food intake

Experiment 1 LBC and HBC groups maintained BC scores throughout the experimental period and had final mean BC scores 2.0 ± 0.00 and 2.9 ± 0.09 respectively. Mean liveweight was consistently lower in LBC than

HBC animals, with final mean liveweights of 43.1 ± 0.94 and 62.4 ± 1.49 kg respectively ($P<0.001$; Fig. 1). The mean daily food intake required to maintain liveweight was lower for the LBC than HBC group (12.4 ± 1.27 vs 18.8 ± 1.25 g DM/kg liveweight respectively, $P<0.01$; Fig. 1).

Experiment 2 LI sheep maintained the same BC score throughout (mean final BC score 2.4 ± 0.03). The mean BC score of the HI animals increased from 2.0 ± 0.01 to 2.5 ± 0.00 . The mean liveweight of the LI group was maintained throughout the study period (final liveweight 56.7 ± 1.16 kg), whereas that of the HI group increased during the 4 week period from 42.8 ± 1.37 to 53.7 ± 1.39 kg at killing. The mean daily food intake of the HI group was consistently higher than that of the LI group, with mean daily intakes over the final week of 37.3 ± 3.32 and 18.9 ± 1.38 g DM/kg liveweight respectively ($P<0.001$) (Fig. 1).

Plasma and CSF profiles

Experiment 1 Compared with LBC sheep, HBC sheep had higher mean plasma glucose ($P<0.01$), insulin ($P<0.01$) and leptin ($P<0.01$), and similar concentrations of NEFA (Fig. 1). In both groups there was no change over time for glucose and NEFA concentrations, and no significant treatment × time interaction for glucose, insulin and NEFA (Fig. 1). Plasma concentrations of insulin increased over time in both HBC ($P<0.01$) and LBC ($P<0.05$) groups (Fig. 1). Plasma leptin concentrations decreased over time in both HBC ($P<0.05$) and LBC ($P<0.05$) animals and there was a significant treatment × time interaction ($P<0.01$) (Fig. 1). Mean CSF concentrations of glucose and insulin were not significantly different between HBC and LBC sheep, and leptin concentrations were below the detection limit of the assay (Table 1).

Experiment 2 There were no significant differences between treatment groups in overall mean plasma concentrations of glucose, NEFA, insulin and leptin (Fig. 1).

Figure 1 Mean BC score, daily food intake, liveweight, and plasma concentrations of NEFAs, glucose, insulin and leptin (means $\pm$ S.E.M.) in sheep in HBC (■; $n=9$) or LBC (●; $n=7$) and fed a liveweight-maintenance ration (Experiment 1; A–G) and sheep with similar terminal BC scores fed freely (HI (□); $n=9$) or a liveweight-maintenance ration (LI (○); $n=7$) (Experiment 2; H–N).

Table 2 LH secretory parameters (means ± S.E.M.) for sheep fed at liveweight maintenance with HBC or LBC (Experiment 1) and sheep with similar terminal BC scores fed freely (HI) or a liveweight-maintenance ration (LI) (Experiment 2)

	Experiment 1		Experiment 2	
	HBC (n=9)	LBC (n=7)	LI (n=7)	HI (n=9)
Mean LH (ng/ml)	2.8 ± 0.36	3.1 ± 0.75	2.3 ± 0.22	3.7 ± 0.49*
Pulse frequency (pulses/7.5 h)	2.3 ± 0.28	2.7 ± 0.42	1.9 ± 0.34	2.9 ± 0.51(*)
Pulse amplitude (ng/ml)	1.3 ± 0.22	1.5 ± 0.47	0.9 ± 0.30	2.1 ± 0.31**
Baseline LH (ng/ml)	2.1 ± 0.31	2.1 ± 0.61	1.8 ± 0.22	3.1 ± 0.40**

Within experiments, (*)*P*<0.10, **P*<0.05, ***P*<0.01.

However, in the HI group, there were significant increases over time in mean circulating glucose (*P*<0.001), insulin (*P*<0.001) and leptin (*P*<0.05) (Fig. 1). There was no change over time in LI sheep and so there were significant interactions between treatment and time for plasma glucose (*P*<0.001), insulin (*P*=0.001) and leptin (*P*<0.01). NEFA concentrations showed no significant change over time and no treatment × time interaction (Fig. 1). Mean CSF concentrations of glucose and insulin were not significantly different between HI and LI sheep, and leptin concentrations were below the detection limit of the assay (Table 1).

LH secretion

Experiment 1 Mean LH, LH pulse frequency, pulse amplitude and basal LH concentrations did not differ significantly between LBC and HBC groups (Table 2).

Experiment 2 HI animals exhibited higher mean LH (*P*<0.05), higher mean LH pulse amplitude (*P*<0.01) and higher mean basal LH concentrations (*P*<0.01) than LI animals (Table 2). There was a trend towards higher LH pulse frequency in HI than LI animals (*P*<0.10) (Table 2).

Hypothalamic gene expression

All mRNA species examined were localised to the ARC, with OB-Rb also being found in the ventromedial hypothalamus. Sense probes showed no hybridisation. Quantification was carried out only in the ARC.

Experiment 1 Compared with the HBC group, LBC sheep had higher levels of gene expression in the ARC for OB-Rb (*P*<0.05), NPY (*P*<0.01) and AGRP (*P*<0.05), lower amounts of CART (*P*<0.05), and similar amounts of POMC mRNA (Figs 2 and 3).

Experiment 2 There were no differences in ARC gene expression between the groups for OB-Rb, NPY, CART and POMC, but AGRP gene expression was higher in HI than LI animals (*P*<0.05) (Fig. 3).

Discussion

The two prescribed paradigms of contrasting nutritional status were achieved in Experiments 1 and 2. The increased LH output (and by inference GnRH output) observed with high vs low food intake (Experiment 2) and not in HBC vs LBC score (Experiment 1) agreed with previous findings (Rhind *et al.* 1985, 1986, 1989, 1991, Rhind & McNeilly 1986). The present data support our hypothesis that in addition to these differential effects on GnRH secretion, there were differential effects on hypothalamic gene expression in the ARC for orexigenic and anorexigenic neuropeptide systems. Overall, the results suggest that the hypothalamus is able to differentiate between constant and increasing nutritional feedback (perhaps mediated by leptin and insulin) as evidenced by the differential responses shown by both hypothalamic appetite-regulating and reproductive neuroendocrine pathways.

The contrast in nutritional state in Experiment 1 lay in the differential steady-state adiposity between the groups. Food intake was functionally similar since it was restricted on an individual basis to the amount required to maintain liveweight for the 4 weeks of experiment and, as indicated by circulating NEFA values, fat mobilisation was insignificant in both groups. Plasma concentrations of glucose, insulin and leptin were all consistently higher in the higher adiposity (HBC) than in the lower adiposity (LBC) sheep, in agreement with previous findings (McCann *et al.* 1992, Blache *et al.* 2000*c*, Delavaud *et al.* 2000, Marie *et al.* 2001).

The contrast in nutritional state achieved in Experiment 2 was not solely represented by the terminal state of different level of food intake at the same level of adiposity. The nutritional state in the 4 weeks preceding killing had been dynamically increasing in the freely-fed (HI) sheep but had been relatively constant in the maintenance-fed (LI) sheep. Again fat mobilisation was negligible in both groups, as evidenced by their similar plasma NEFA concentrations, but plasma concentrations of glucose, insulin and leptin increased in the HI sheep during the 4 week study to reach concentrations similar to the steady-

Figure 2 Representative autoradiographic images of hypothalamic ARC gene expression for NPY, AGRP, POMC, CART and OB-Rb in sheep fed a liveweight-maintenance ration with HBC (A, C, E, G, I) or LBC (B, D, F, H, J; Experiment 1). 3V, third ventricle; VMH, ventromedial hypothalamus. Bar=3 mm.

state values recorded in the LI sheep throughout the study. Thus, at the time of killing, the key difference in the signal provided by circulating levels of insulin and/or leptin to the hypothalamus lay not in the concentration of hormone on that day but in the preceding pattern of feedback.

The extent to which systemic feedback to the ARC is exerted through changes in CSF concentrations is uncertain. Although glucose, insulin and leptin have all

Figure 3 Hypothalamic ARC gene expression (means $\pm$ S.E.M.) for NPY, AGRP, POMC, CART and OB-Rb in (A) sheep with HBC (open bars) or LBC (solid bars) and fed a liveweight-maintenance ration (Experiment 1) and (B) sheep with similar terminal BC scores fed freely (HI; striped bars) or a liveweight-maintenance ration (LI; dotted bars) (Experiment 2). * $P<0.05$, ** $P<0.01$.

been detected in ovine CSF (Miller *et al.* 1998, Blache *et al.* 2000c), there were no treatment differences in glucose and insulin concentrations in the CSF in this study, irrespective of differences in concentrations in plasma, and leptin values were below detection. However, it is acknowledged that in Experiment 2 critical progressive changes as seen in the plasma concentrations of these factors (Fig. 1) may have occurred in the CSF but remained undetected.

Studies of gene expression of hypothalamic orexigenic and anorexigenic neuropeptides have most commonly been undertaken in rodents following acute challenge paradigms such as fasting or food restriction. In sheep the relatively few such studies have been carried out using chronic food restriction paradigms (McShane *et al.* 1993, Adam *et al.* 1997, Henry *et al.* 2000) or fasting (Adam *et al.* 2002). The effects on hypothalamic gene expression of food intake and adiposity have not previously been dissociated. Long-term alterations in adiposity were studied previously by Henry *et al.* (2000) but their model differs fundamentally from the present one in that their

'fat' sheep continued to feed freely (and were therefore satiated) while the 'thin' counterparts had their intake restricted to liveweight maintenance. Sheep in our differential adiposity model (Experiment 1) are all restricted to the same (liveweight maintenance) level of intake for the 4 weeks prior to killing. Furthermore, our model presents a more modest adiposity differential (20 vs 29% body fat) than that of Henry *et al.* (2000) (15 vs 36% body fat). Nonetheless, results from the two models are in general agreement, with increased NPY and AGRP and no difference in POMC gene expression between thin and fat sheep (Henry *et al.* 2000, 2001*b*); here, we additionally report elevated OB-Rb and reduced CART gene expression in the ARC of thin vs fat sheep. Hypothalamic gene expression in sheep has not previously been reported for the contrasting paradigm of differential food intake at the same level of adiposity (Experiment 2). In this scenario, the only difference observed was a higher level of AGRP gene expression in the ARC of HI vs LI sheep.

Differences in ARC gene expression between HBC and LBC animals (Experiment 1) were largely consistent with putative induction by the differences in circulating insulin and leptin. NPY mRNA expression was lower in HBC than LBC animals and this was associated with higher plasma concentrations of insulin and leptin, both of which have been shown to suppress NPY mRNA when administered i.c.v. (Schwartz *et al.* 1992, Henry *et al.* 1999). NPY mRNA co-localises with OB-Rb in the ovine ARC (Williams *et al.* 1999), and the simultaneous up-regulation of both mRNAs in LBC sheep is suggestive of a leptin-driven response (Ahima *et al.* 2000). The absence of differences in gene expression for both OB-Rb and NPY between HI and LI sheep (Experiment 2) was consistent with their similar terminal BC scores and plasma leptin and insulin concentrations. However, since their initial BC scores were clearly different, extrapolating from the results of Experiment 1, it is postulated that NPY and OB-Rb gene expression were initially higher in HI than LI animals. Levels of expression then steadily declined in HI sheep inversely associated with the circulatory increases in insulin and leptin, so that they were similar to those in LI sheep by the end of the study period.

AGRP gene expression, like NPY gene expression, was higher in LBC than HBC animals (Experiment 1), which was consistent with suppression by the increased circulating leptin (Wilson *et al.* 1999). However, unlike NPY gene expression, AGRP gene expression in the ARC was higher in HI than LI animals (Experiment 2) despite their similar final plasma concentrations of insulin and leptin. Possible reasons are: (i) gene expression for AGRP was unresponsive to the increasing circulating concentrations of insulin and leptin, (ii) AGRP had a greater response time than NPY to the circulatory changes in insulin and leptin, (iii) there was a greater initial level of expression in animals of the HI group, attributable to their low initial

BC, and expression was still decreasing after 4 weeks, or (iv) AGRP gene expression is regulated by other factor(s). Since AGRP and NPY mRNAs are co-expressed within the ARC of rodents (Mercer *et al.* 2000), findings from the present study suggest either that there was transcript-specific regulation within NPY/AGRP-expressing neurons or that different neuronal sub-populations were activated.

AGRP antagonises the actions of the POMC products α MSH and γ MSH at their brain receptors, thus mediating orexigenic actions by suppressing the melanocortin feedback pathway (Cone 1999). However, POMC gene expression was not affected by BC or food intake in this study. This is consistent with the previous report that long-term alterations in adiposity and intake did not affect hypothalamic POMC expression in ovariectomised ewes (Henry *et al.* 2000). Conversely CART mRNA, which extensively co-localises in the ARC with POMC mRNA (rodent: Elias *et al.* 1998, sheep: Adam *et al.* 2002), was up-regulated in HBC vs LBC sheep in Experiment 1, unlike the results of Henry *et al.* (2001*b*); this is also suggestive of transcript-specific regulation within single neurons. Leptin concentrations were different between the groups in Experiment 1, but not at the time of killing in Experiment 2. The apparent sensitivity of CART gene expression to the steady-state level of adiposity (Experiment 1) but not to differing levels of food intake (Experiment 2) was therefore perhaps indicative of its acute sensitivity to the amount of leptin feedback (Kristensen *et al.* 1998) and of its rapid response to changes in circulating leptin.

There is increasing support for the concept of body-weight regulation incorporating an 'appropriate body-weight' target which is encoded within the brain, against which nutritional feedback is compared, and responses are integrated within the hypothalamus (Mercer & Speakman 2001). Thus, it is postulated that feedback in underweight individuals activates orexigenic hypothalamic pathways to attempt to restore appropriate bodyweight, and in overweight individuals activates anorexigenic systems. On the basis of the observed patterns of neuropeptide expression, it is suggested that the hypothalamus of LBC sheep in Experiment 1 perceived that bodyweight was substantially sub-optimal; appropriate orexigenic pathways (NPY, AGRP) were therefore activated and anorexigenic pathways (CART) were suppressed to attempt to restore optimum bodyweight. Conversely the HI sheep in Experiment 2 had achieved similar bodyweight to their LI counterparts at killing, so that activities of the orexigenic NPY and anorexigenic CART pathways had equalised between the groups. Only AGRP remained differentially expressed, for reasons that are open to conjecture.

The extent to which observed differences in hypothalamic gene expression were likely to have been involved in mediating effects on GnRH secretion in the present study is open to speculation. Indeed, anomalies are

revealed in putative neuropeptide–GnRH relationships. Thus, increased ARC gene expression for NPY was not associated with decreased reproductive neuroendocrine activity in Experiment 1, in contrast to McShane *et al.* (1993) and Adam *et al.* (1997), and increased AGRP gene expression was not associated with increased GnRH/LH output, in contrast to the findings of Stanley *et al.* (1999). However, GnRH/LH output was stimulated in the group exhibiting elevated AGRP gene expression in Experiment 2, which would be consistent with the findings of Stanley *et al.* (1999). It is therefore possible that either the relative activity of these neuropeptide systems is the critical factor determining GnRH secretion patterns or that alternative unknown mediators are involved. Nonetheless, synaptic contacts are known to exist that indicate interactive capability between GnRH cells and NPY and POMC/CART neurons (Tillet *et al.* (1989) and Elias *et al.* (1998) respectively). The anomalies may potentially be explained by changes in gene expression over time, driven perhaps by the circulatory changes in insulin and leptin, which might be critical in dictating the GnRH response.

In conclusion, the present models have demonstrated contrasting hypothalamic responses to steady-state adiposity as opposed to an increase in food intake, in terms of both reproductive neuroendocrine activity and gene expression for hypothalamic appetite-regulating neuronal systems. However, it is clearly difficult to dissociate completely the systemic signal(s) from intake and from adipose tissue since changes in intake over time lead to changes in adiposity. Here, circulating concentrations of leptin and insulin appeared to reflect both food intake and adiposity, providing a composite signal of nutritional status in both models. Interpretation is further confounded by the ability to determine ongoing metabolic endocrine status over time but to examine hypothalamic gene expression only at a single time point. Nonetheless, the observed differences in hypothalamic gene expression for the appetite-regulating neuropeptides, with the exception of AGRP, were consistent with the contemporary level of nutritional feedback provided by these hormones. However, the difference in GnRH/LH response between the models was not consistent with mediation by the level of contemporary nutritional feedback *per se* but by its state of change; thus, increased nutritional feedback was stimulatory but constant high feedback was not. This suggests that the GnRH response to nutrition results from computation within the hypothalamus of dynamic changes in nutritional feedback, which may involve the appetite-regulatory pathways studied or alternative intermediary neuronal mechanisms. Overall, the GnRH response demonstrates the ability of the hypothalamus to retain a nutritional memory that can influence subsequent responses to nutritional feedback, with potential relevance for the treatment of appetite/bodyweight disorders such as obesity as well as infertility.

Acknowledgements

This research was funded by Scottish Executive Environment and Rural Affairs Department. Z A A was in receipt of a Boyd Orr Research Centre studentship. We thank Drs N Hoggard, J Mercer, A Ross, P Barrett and S Sabol for probes, M Annand for NEFA and glucose analyses, F Gebbie for oestradiol analyses, T Atkinson for iodinations, N Hoggard for assistance with the leptin antibody, Macaulay Land Use Research Institute Glensnaugh Research Station animal house staff for daily animal care, the National Institute of Diabetes and Digestive and Kidney Diseases and the Scottish Antibody Production Unit for RIA materials and G Horgan of Biomathematics & Statistics Scotland for statistical advice.

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Received 22 May 2002

Accepted 23 July 2002