Seasonal and dose-dependent effects of intracerebroventricular leptin on LH secretion and appetite in sheep

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Abstract

The role of leptin in neuroendocrine appetite and reproductive regulation remains to be fully resolved. A series of three experiments was conducted using adequately nourished oestradiol-implanted castrated male sheep. In a cross-over design ($n=6$), responses to a single i.c.v. (third ventricle) injection of leptin (0.5, 1.0 and 1.5 mg ovine leptin (oLEP) and 1.0 mg murine leptin (mLEP)), N-methyl-D-aspartate (NMDA, 20 μ g) or 0.9% saline (control) were measured in terms of LH secretion (4 h post-injection compared with 4 h pre-injection) and appetite (during 2 h post-injection) in autumn (Experiment 1). NMDA and 1.0 mg oLEP treatments were repeated in the same sheep in the following spring (Experiment 2). With an additional 12 sheep ($n=18$ in cross-over design), responses to low-dose 'physiological' i.c.v. infusion of leptin (8 ng/h for 12 h daily for 4 days), insulin (0.7 ng/h) and artificial cerebrospinal fluid were measured in the next spring (Experiment 3). LH was studied over 8 h and appetite over 1 h on days 1 and 4 of infusion. In Experiment 1 (autumn), oLEP overall increased LH pulse frequency by up to 110% ($P<0.05$), decreased LH pulse amplitude ($P<0.05$) and decreased appetite ($P<0.05$). mLEP reduced LH pulse amplitude ($P<0.05$) without

significant effect on appetite, while NMDA reduced appetite ($P<0.05$) but had no effect on LH. In Experiment 2 (spring), LH responses were 'surge-like' with highly significant increases in the moving average LH concentration after 1.0 mg oLEP ($P<0.001$) and after NMDA ($P<0.001$). Compared with similar analysis of Experiment 1 results, the LH response in spring was greater than that in autumn for both 1.0 mg oLEP ($P<0.05$) and NMDA ($P<0.005$). Conversely, unlike in autumn (Experiment 1), there was no effect of 1.0 mg oLEP or NMDA on appetite in the spring (Experiment 2). In Experiment 3 (spring), 'physiological' i.c.v. infusion of oLEP or insulin increased LH pulse frequency by up to 100% ($P<0.001$) compared with the control infusion on both days 1 and 4, but there were no effects on appetite. These results indicate that intracerebral leptin both stimulates reproductive neuroendocrine output and decreases appetite in adequately nourished sheep. However, the responses of these two axes were dose-dependent and differentially affected by the time of year, suggesting dissociation of the neural pathways involved.

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Introduction

The role of leptin in neuroendocrine regulation remains to be fully resolved. Although it has clearly been shown to act as a satiety factor within the hypothalamus, and acutely reduced circulating leptin levels with fasting evoke a powerful orexigenic drive, its role as a metabolic signal to the reproductive neuroendocrine axis is less clear (for review see Ahima *et al.* 2000). It appears that leptin plays a permissive action in this latter role, acting as a metabolic gate or threshold rather than a precise trigger to the gonadotrophin-releasing hormone (GnRH) pulse

generator. This hypothesis is supported by experiments in which leptin restores pulsatile luteinising hormone (LH) secretion in fasted rats and sheep (Nagatani *et al.* 1998, 2000), although direct leptin stimulation of LH *in vivo* has not been demonstrated in satiated animals. Pituitary LH secretion, which is strictly regulated by GnRH and which is readily measured in circulating blood, is commonly used as an *in vivo* measure of hypothalamic GnRH output (Clarke & Cummins 1982).

Central leptin administration (i.c.v.) reduced food intake but did not affect LH secretion in adequately nourished ovariectomised ewes (Henry *et al.* 1999), but

restored LH secretion in food-restricted ovariectomised ewes without affecting food intake (Henry *et al.* 2001). Morrison *et al.* (2001) also found that central leptin administration reduced food intake in adequately nourished and not in food-restricted ovariectomised ewes, but reported no effect on LH secretion in either group. Finally, Blache *et al.* (2000a) found that central leptin administration in adequately nourished intact rams decreased both food intake and LH secretion. The over-riding intake-depressing effect of leptin in adequately fed sheep in the above paradigms may have masked any potential GnRH-stimulating effect, i.e. GnRH stimulation was only observed when leptin was given to undernourished, and presumably leptin-deficient, animals. The compatibility of these data may be further confounded by the different times of year in which the studies were carried out, since a seasonal difference in sensitivity of the appetite axis to centrally administered leptin has since been reported for gonadectomised sheep (Clarke *et al.* 2001).

With the advent of assays able to detect leptin in cerebrospinal fluid (CSF), it is reported that the 'normal' or physiological concentration range of leptin in sheep CSF is about one-tenth to one-twentieth of the plasma concentration range (Blache *et al.* 2000b). In our experience, CSF from sheep across a range of adiposity and food intake has leptin concentrations ranging from 0.3 to 1.0 ng/ml (C L Adam & M Marie, unpublished data using the assay of Marie *et al.* 2001). This equates to about one-tenth of the plasma concentration in 'lean' sheep with low intake ranging to 'fat' sheep with high food intake found previously by our group (Marie *et al.* 2001). The majority of studies that have employed central administration of leptin in order to elucidate its neuroendocrine role have used doses that may have produced intra-hypothalamic concentrations considerably in excess of the normal physiological range. For example, the central infusion doses employed in the foregoing studies in sheep varied from around 40 µg/h down to 40 ng/h (Henry *et al.* 1999, 2001, Blache *et al.* 2000a, Morrison *et al.* 2001). Effects of leptin on feed intake and GnRH secretion may be concentration dependent. Although no studies have directly addressed this issue, there is support for this hypothesis with the finding that the hypothalamic neuronal targets of leptin mediating recovery from starvation are apparently distinct from those that are activated by mild hyperleptinaemia (Ahima *et al.* 1999). Furthermore, a theoretical framework for the above postulate can be borrowed from the actions of insulin on feed intake and GnRH secretion. In rats, systemic doses of insulin that were high enough to cause hypoglycaemia stimulated feeding (Friedmann & Granneman 1983) whereas sub-hypoglycaemic insulin doses suppressed feeding (Vanderweele *et al.* 1980). Moreover, effects of insulin on feed intake were dose-dependent when it was infused into the cerebral ventricles (Brief & Davis 1984). In terms of effects at the level of the GnRH pulse-generator, Hileman *et al.* (1993) found that a single i.c.v. injection of

insulin in sheep (500 ng–500 µg) decreased LH secretion, whereas we have previously shown that a continuous infusion of insulin at a physiological dose of 570 pg/h increased LH pulse frequency (Miller *et al.* 1995).

In the present study, a series of three experiments was conducted using the oestradiol-implanted male castrated sheep model (Adam & Findlay 1998, Nagatani *et al.* 2000). This model produces constant physiological concentrations of gonadal steroid feedback, obviating the effects of seasonal fluctuations in circulating concentrations. This provides tonic restraint to GnRH/LH pulsatility without which stimulation of this axis would be harder to detect. Centrally administered (third cerebral ventricle, i.c.v.) N-methyl-D-aspartate (NMDA) was used as a positive control for GnRH/LH stimulation since it is a recognised secretagogue for GnRH and the magnitude of the response is photoperiod dependent in sheep (Viguie *et al.* 1995). The objectives were to determine whether a single i.c.v. injection of leptin would stimulate GnRH/LH before the response is confounded by the GnRH-suppressive effect of a leptin-induced reduction in food intake (Experiment 1), whether the responses of both the appetite and GnRH/LH axes were affected by the time of year within the ovine breeding season (Experiment 2), and whether the responses of both of these axes to a low 'physiological' i.c.v. infusion dose of leptin differed from previous 'pharmacological' paradigms (Experiment 3).

Materials and Methods

All experimental procedures involving animals were conducted under the authority of the Animals (Scientific Procedures) Act of 1986 and received prior approval from the local Ethical Review Committee.

Animals and i.c.v. cannulation

All sheep were Suffolk × Greyface adult male castrates (approximately 1 year old at surgery) with average live weight 52 ± 1.0 kg and body condition score 2.4 ± 0.05 (after Russel *et al.* 1969). They were housed in individual pens in natural lighting in Aberdeen (52°N) and given a complete chaff-based diet twice daily at 0800 h and 1700 h in amounts designed to maintain live weight (maintenance fed). Water was provided *ad libitum*. They were acclimatised to these conditions and to frequent human contact prior to surgery. Cannulation of the third cerebral ventricle, as reported by Miller *et al.* (1995), was undertaken in 24-h fasted sheep maintained on halothane anaesthesia (2% halothane; Concord Pharmaceuticals Ltd, Dunmow, Essex, UK). While under general anaesthesia, each sheep was given two subcutaneous oestradiol-containing implants made from Silastic tubing (Adam & Findlay 1998) which raised plasma oestradiol concentrations to a steady 3.93 ± 0.45 pg/ml (measured during

each experiment by radioimmunoassay; Mann *et al.* 1995). Immediately before surgery, sheep received antibiotics (ampicillin 500 mg/ml; Penbritin; SmithKline Beecham, and gentamicin 50 mg/ml; Pangram 5%; Bimeda UK, Llangefni, Anglesey, UK) and analgesics (carprosen 50 mg/ml; Rimadyl; Pfizer and buprenorphine 0.3 mg/ml; Temgesic; Schering-Plough Ltd, Welwyn Garden City, Herts, UK). Antibiotic (gentamicin) was given daily for 3 days and analgesia (buprenorphine) was given as required. The sheep were allowed a minimum post-operative recovery period of 4 weeks prior to experimentation.

Experiment 1

In October–November 1999 (late autumn), six surgically prepared sheep were given a single 0.1 ml injection into the third cerebral ventricle (i.c.v.) of each of the following in a balanced cross-over (Latin square) design, with 1-week intervals between treatments: 0.9% saline negative control, or (dissolved in 0.9% saline) 0.5 mg recombinant ovine leptin (0.5 oLEP, supplied by Arie Gertler; Gertler *et al.* 1998), 1.0 mg ovine leptin (1.0 oLEP), 1.5 mg ovine leptin (1.5 oLEP), 20 µg NMDA positive control (NMDA; Sigma International, Poole, Dorset, UK) or 1.0 mg recombinant murine leptin (1.0 mLEP; courtesy of NIH). The NMDA dose rate was calculated from published effective doses for i.v. administration in sheep (Viguie *et al.* 1995) and for i.c.v. administration in rhesus monkeys (Gay *et al.* 1993). Starting at 0800 h, blood was taken via a temporary jugular catheter every 15 min for 4 h before and 4 h after i.c.v. injection and plasma was stored at –20 °C prior to LH analysis. Voluntary food intake (VFI) was determined by measuring the *ad libitum* consumption of the chaff-based diet during the 2-h period immediately post-injection.

Experiment 2

In April 2000 (spring), the NMDA and 1.0 oLEP treatments of Experiment 1 were repeated in each of the same six sheep, with a 1-week interval between cross-over of treatments. Blood was taken via a jugular catheter every 15 min for 4 h before and 4 h after i.c.v. injection, and plasma LH and VFI were measured as before. Control VFI measurements were obtained in all sheep on two occasions in the week prior to the start of the experiment; during the 2-h test period the sheep were handled every 15 min to simulate blood sampling.

Experiment 3

In March–April 2001 (spring), the same six sheep of Experiments 1 and 2, together with 12 additional surgically prepared steroid-replaced male castrates, were allocated into two equal groups. During the 6 weeks of

Experiment 3, nine sheep per week were undergoing treatment whilst the other nine had a 1-week rest interval. Each sheep was given each of three infusion treatments in a randomised cross-over design. Infusions into the third ventricle were performed for 12 h/day (0800–2000 h) for 4 consecutive days at a flow rate of 5 µl/min via a battery-powered syringe driver (Graseby Medical Ltd, Watford, Herts, UK) attached to a harness on the animal's back. The infusates were prepared in a carrier of artificial CSF (aCSF) as described by Nilsson *et al.* (1991) and which is similar in composition to sheep CSF (Pollay *et al.* 1972). The infusion treatments were: aCSF control, porcine insulin in aCSF (0.7 ng/h; biopotency = 24.0 IU/mg; Sigma International) or ovine leptin in aCSF (8.0 ng/h). The insulin infusion treatment acted as a positive control as it had been previously shown by Miller *et al.* (1995) to stimulate LH secretion in male sheep when infused into the third cerebral ventricle using the same infusion protocol.

Jugular catheters were inserted the day before the start of i.c.v. infusion and remained in place for blood sampling during the 4-day infusion. On days 1 and 4 of infusion, starting at 0800 h, blood samples were taken every 15 min for 8 h for LH analysis, and VFI was determined by measuring the *ad libitum* consumption of the chaff-based diet during a 1-h period from 1400 h to 1500 h.

Calculation of insulin and leptin infusion doses in Experiment 3

The dose and infusion rate for insulin, derived from CSF insulin parameters described by Davson *et al.* (1987), had previously produced physiological increases in ovine CSF insulin concentrations (Miller *et al.* 1995). The dose and infusion rate for leptin were calculated using the same parameters with the inclusion of the basal leptin concentration in sheep CSF. The dose for leptin was designed to produce a constant concentration in the third ventricle of approximately four times the physiological level for a 'maintenance-fed' sheep (about 0.3 ng/ml) to values approximating a 'well-fed' sheep (about 1.2 ng/ml) for the duration of the 12-h infusion per day.

LH radioimmunoassay

LH concentrations were determined in duplicate aliquots of plasma samples with the assay technique of Adam *et al.* (1998) using radioimmunoassay reagents provided by NIDDK (Rockville, MD, USA) and expressed in terms of the reference standard NIDDK-oLH-1–2. Intra- and interassay coefficients of variation averaged 5 and 10% respectively, and the detection limit was 0.05 ng/ml. The effect of the interassay variation on comparisons of LH pulse variables between treatments, and between Experiments 1 and 2, was avoided by assaying all samples from one animal in the same assay run.

Figure 1 (a) LH pulse frequency, (b) LH pulse amplitude, (c) mean LH concentration and (d) VFI of steroid-replaced castrated male sheep ($n=6$) after a single i.c.v. (third ventricle) injection of 0.9% saline (CONT; open bars), 0.5 oLEP (light grey bars), 1.0 oLEP (dark grey bars), 1.5 oLEP (solid bars), 20 µg NMDA (hatched white bars) or 1.0 mLEP (hatched grey bars) (Experiment 1; autumn). Values are means $\pm$ S.E.M. * $P<0.05$ compared with CONT.

LH pulse analysis

The LH values from the serial samples were analysed with a modified version of the 'Pulsar' algorithm developed by Merriam & Wachter (1982) and modified for the Apple Macintosh computer ('Munro'; Zaristow Software, Haddington, East Lothian, UK). The G parameters (the number of standard deviations by which a peak must exceed the baseline in order to be accepted; G_1 – G_5) were set at 3, 2.5, 1.9, 1.2 and 0.9, these being the requirements for pulses composed of one to five samples that exceed the baseline respectively. The Baxter parameters describing the parabolic relationship between the concentration of hormone in a sample and the standard deviation (assay variation) about that concentration were 0.25132 (b_1 , the y intercept), 0.06557 (b_2 , the x coefficient) and 0.00291 (b_3 , the x^2 coefficient). Pulse frequency, mean pulse amplitude and mean concentration of LH were calculated for each profile.

Statistical analysis

LH pulse frequency data were tested for homogeneity of variance and were found to be normally distributed. ANOVA was therefore used to compare treatment effects on feed intake, LH pulse frequency, LH pulse amplitude and mean LH concentration, accounting for individual animal variation and the cross-over design. When the main effect of the infusion treatments was significant

($P<0.05$), post-hoc one-way ANOVA was used to test for differences between specific treatments. In Experiment 2, identification of individual LH pulses after treatment was difficult due to the surge-like nature of the response (Fig. 2b), with LH pulses tending to merge with each other. Pulse interactions were therefore removed by calculating the moving average of five consecutive samples (Fig. 2d) and repeated measures ANOVA was conducted on these moving average values.

Results

Experiment 1

There was no difference between treatment groups prior to injection, and no difference between the pre- and post-injection values in the control group, for any of the LH pulse variables measured. Overall, the ovine leptin treatments increased LH pulse frequency ($P<0.05$) and decreased pulse amplitude ($P<0.05$; Fig. 1). Specifically, the 0.5 oLEP and 1.0 oLEP treatments significantly ($P<0.05$) increased LH pulse frequency by 110% and 70% respectively, compared with the negative control injection (CONT), whereas the 72% increase after 1.5 oLEP failed to reach significance (Fig. 1a). The 0.5 oLEP and 1.5 oLEP treatments significantly ($P<0.05$) reduced pulse amplitude by 42% and 45% respectively, compared with the CONT treatment, whereas the response to 1.0 oLEP

was not significant (Fig. 1b). None of the ovine leptin treatments altered the mean LH concentrations (Fig. 1c). The murine leptin (1.0 mLEP) reduced pulse amplitude by 47% ($P<0.05$) but had no effect on the other LH pulse variables. The positive control (NMDA) treatment had no significant effect on any of the LH pulse variables.

Overall, the ovine leptin treatments reduced VFI ($P<0.05$; Fig. 1d). Specifically, 1.5 oLEP and 1.0 oLEP ($P<0.05$) reduced VFI by 35% and 28% respectively, compared with the CONT treatment, whereas 0.5 oLEP had no significant effect. The NMDA treatment reduced VFI by 31% ($P<0.05$), compared with the CONT treatment, but the 32% reduction after 1.0 mLEP treatment failed to reach significance.

Experiment 2

There was no difference in any of the LH pulse variables between groups in the 4 h before treatment (Fig. 2a). Neither 1.0 oLEP nor NMDA significantly altered any of the LH variables compared with pre-injection values, although there was an apparent trend for both 1.0 oLEP and NMDA to increase LH pulse amplitude ($P=0.09$) and mean concentration ($P=0.06$). However, examination of individual pulse profiles revealed that LH pulses were tending to merge together into a 'surge-like' response post-injection, making identification of individual pulses both difficult and inappropriate (Fig. 2b). A better representation of the effects of 1.0 oLEP and NMDA was found by calculating a moving average of five consecutive samples of LH concentration in order to smooth the pulse effect (Fig. 2d). Repeated measures ANOVA indicated that there was a highly significant increase in the moving average LH concentration after 1.0 oLEP ($P<0.001$) and NMDA ($P<0.001$) i.c.v. injections. Repeated measures analysis also revealed a significant effect of time of year on the moving average LH concentration when comparing the 1.0 oLEP ($P<0.05$) and NMDA ($P<0.005$) treatments between Experiments 1 and 2 (Fig. 2d).

Although there was no saline-injected (control) treatment in this experiment, the effect on VFI of the 1.0 oLEP and NMDA treatments was compared with control VFI measurements obtained during the week before i.c.v. injections commenced (Fig. 2c). There was no effect detected of either of the i.c.v. treatments on VFI. However comparison with VFI values obtained in Experiment 1 revealed a significant effect of time of year on VFI responses to the 1.0 oLEP ($P<0.05$) and NMDA ($P<0.005$) treatments (Fig. 2c).

Experiment 3

Overall, the leptin and insulin treatments increased LH pulse frequency ($P<0.0001$) with no effect on any of the other LH pulse variables (Figs 3 and 4). Specifically, on day 1 the leptin ($P<0.01$) and insulin ($P<0.05$) infusion

treatments increased LH pulse frequency by 90% and 55% respectively, compared with the control infusion. On day 4, the leptin ($P<0.005$) and insulin ($P<0.01$) infusion treatments increased LH pulse frequency by 100% and 95% respectively. There was no effect of either the leptin or insulin infusion treatments on VFI (Fig. 3d).

Discussion

Our data strongly support the hypothesis that intracerebral leptin is a metabolic modulator of not only appetite, but also of GnRH secretion. The results have demonstrated for the first time the stimulation of LH pulsatility by physiological amounts of leptin infused into the third cerebral ventricle of adequately nourished sheep, with no simultaneous effect on appetite. Although the stimulatory effect of leptin was manifest as an increase in LH pulse frequency, this is indicative of activation of the hypothalamic GnRH neurones (Clarke & Cummins 1982). In addition, the results demonstrate that responses to a single pharmacological i.c.v. leptin injection are seasonally dependent yet distinct for the appetite and reproductive neuroendocrine axes.

Previous *in vivo* demonstrations of LH stimulation by leptin have been confined to the restoration of LH secretion in sheep that were fasted or undernourished, and therefore presumably leptin deficient (Nagatani *et al.* 2000, Henry *et al.* 2001). The failure to demonstrate LH stimulation by i.c.v. leptin in previous studies of adequately fed sheep (Henry *et al.* 1999, 2001, Blache *et al.* 2000b, Morrison *et al.* 2001) could be explained by the design of these experiments such that the over-riding intake-depressing effect of leptin over the 3- to 8-day infusion protocols counteractively inhibited GnRH secretion. In support of our original hypotheses, by looking at immediate responses to a single i.c.v. injection of leptin we were able to demonstrate LH stimulation despite a transient reduction in appetite (Experiment 1) and, moreover, by employing a much lower 'physiological' i.c.v. infusion dose of leptin we were able to detect LH stimulation with no concurrent effect on appetite (Experiment 3). The intriguing findings of the present studies with respect to LH stimulation by i.c.v. leptin was the difference in the magnitude of the response at the different times of the year and the difference in the nature of the response to 'physiological' as opposed to 'pharmacological' administration. Thus, in the late autumn (Experiment 1; short days), LH pulse frequency was stimulated and LH pulse amplitude decreased by a single 'pharmacological' i.c.v. leptin injection, whereas the same treatment in spring (Experiment 2; long days) induced a large surge-like release of LH that masked any increase in pulsatility (Fig. 2b and d); however, a 'physiological' i.c.v. infusion of leptin in the spring did not induce a surge-like response but specifically increased LH pulse frequency (Fig. 4).

Figure 2 (a) LH pulse frequency, LH pulse amplitude and mean LH concentration of steroid-replaced castrated male sheep ($n=6$) after a single i.c.v. (third ventricle) injection of 1.0 oLEP (grey bars) or 20 μ g NMDA (hatched bars) (Experiment 2; spring). Control samples (open bars) were taken for 4 h prior to injection. (b) Representative individual LH profile showing surge-like response after i.c.v. injection of 1.0 oLEP (open symbols indicate significant pulses) (Experiment 2). (c) VFI during 2 h after CONT, 1.0 oLEP and NMDA treatments in Experiments 1 and 2. (d) Smoothed plasma LH profiles (5-day moving average) showing responses to 1.0 oLEP and NMDA in Experiment 1 (autumn, November; solid symbols) and Experiment 2 (spring, April; open circles). Values are means $\pm$ S.E.M. For clarity, S.E.M. values are shown only for every second point in (d). The arrows indicate the time of i.c.v. injection in (b) and (d).

The NMDA treatment was ineffective as a positive control in the autumn (Experiment 1). However, the dose rate was extrapolated from a peripheral dose used in sheep (Viguie *et al.* 1995) and an i.c.v. dose for rhesus monkeys (Gay *et al.* 1993) and may have been sub-optimal at this time of year. Nonetheless, the same dose in the spring was effective (Experiment 2; Fig. 2d), in agreement with the seasonal sensitivity reported for the LH response to

NMDA in sheep (Viguie *et al.* 1995). The reason for a seasonal difference in the GnRH response to leptin is open to speculation. It is unlikely to be attributable to seasonal changes in gonadal steroid concentrations interacting with the actions of leptin (Ainslie *et al.* 2001, Kimura *et al.* 2002) since steroid-clamped oestradiol-implanted castrates were used. However, the similar, seasonally dependent responses of LH secretion and appetite to NMDA in the

Figure 3 (a) LH pulse frequency, (b) LH pulse amplitude, (c) mean LH concentration and (d) VFI of steroid-replaced castrated male sheep ($n=18$) on days 1 and 4 of the following infusion treatments (4 days, 12 h/day) into the third cerebral ventricle: aCSF (CONT; open bars), insulin (INS, 0.7 ng/h; grey bars) or ovine leptin (LEP, 8 ng/h; solid bars) (Experiment 3; spring). Values are means $\pm$ S.E.M. * $P<0.05$, ** $P<0.01$, *** $P<0.005$ compared with CONT.

present study (in agreement with Viguie *et al.* 1995) are consistent with leptin acting in part via glutamatergic pathways. Glutamatergic pathways operate in the hypothalamus to regulate the release of peptides and catecholamines to control the secretion of GnRH/LH (Kumar *et al.* 1993). NMDA receptors are found in regions of the ovine hypothalamus that are known to have important functions in seasonality and control of reproduction and appetite (Anderson *et al.* 1999), and manipulation of glutamatergic neurotransmission affects the rate of photoperiodically regulated sexual maturation in hamsters (Ebling & Cronin 1998). Finally, leptin–NMDA interactions have been reported, at least in the hippocampus, in which leptin facilitated NMDA receptor function by rapidly enhancing NMDA-induced increases in intracellular calcium levels (Shanley *et al.* 2001).

A plausible explanation for the seasonal change in response to leptin is that photoperiod (short daylength) is the main cue for stimulation of GnRH pulsatility in the sheep (e.g. for review see Karsch *et al.* 1984), effectively over-riding additional nutritional stimulation in the autumn. When photoperiod support is waning as the

days lengthen in the spring, there is evidence that the reproductive axis becomes more receptive to nutritional stimulation since improved nutrition at this time delays both anoestrus in ewes (Knight *et al.* 1983) and testicular regression in rams (Martin *et al.* 1994).

Although there was a trend towards negative dose-dependence of the LH pulsatile response within the pharmacological doses of i.c.v. leptin used in Experiment 1, the more striking difference in response was seen between the 'pharmacological' and 'physiological' doses of Experiments 2 and 3 respectively, given at the same time of year. In other words, a modest increase in intracerebral leptin was more stimulatory to the frequency of GnRH pulses than was a large increase in leptin, which induced a greater, surge-like release of GnRH. This is not without precedent since a dose-dependent response exists for the effects of i.c.v. insulin on LH secretion. Hileman *et al.* (1993) found that a large i.c.v. dose of insulin (500 ng or more in a single injection) decreased LH secretion, whereas 'physiological' i.c.v. insulin infusion at 0.7 and 0.6 ng/h stimulated LH secretion in the present trial and in the study of Miller *et al.* (1995) respectively. The

Figure 4 Representative individual LH profiles showing responses in two steroid-replaced castrated male sheep on day 4 of the following infusion treatments (12 h/day) into the third cerebral ventricle: aCSF (control; top two panels), insulin (0.7 ng/h; middle two panels) or ovine leptin (8 ng/h; bottom two panels). The open symbols indicate significant pulses.

similarity of the appetite and LH responses between i.c.v. infusions of insulin and leptin in the present study could be indicative of similar pathways of action. Indeed, insulin may interact with the leptin receptor (OB-Rb), since i.c.v. insulin down-regulates OB-Rb gene expression in the sheep hypothalamus (Daniel *et al.* 2000) and gene expression for both insulin receptor and OB-Rb is found in the same (arcuate) hypothalamic nucleus in sheep (Z A Archer, P A Findlay & C L Adam, unpublished observations; Williams *et al.* 1999).

The seasonal difference in sensitivity of the appetite axis to i.c.v. leptin in the present study using oestradiol-implanted castrated sheep, with intake inhibited by leptin in the autumn but not in the spring, is opposite to the responses reported for gonadectomised sheep (Clarke *et al.* 2001). However, in both studies, the appetite-depressing effect of leptin was greatest when the measured basal voluntary intake was at its lowest. The disparity may be resolved when such studies are conducted in controlled artificial photoperiods rather than in natural photoperiods. Nonetheless, unlike in the present study, the sheep used by Clarke *et al.* (2001) did not have gonadal steroid replacement and it is suggested that oestradiol is required to facilitate leptin actions in the brain (Bennett *et al.* 1998, Pelkeymounter *et al.* 1999, Ainslie *et al.* 2001, Kimura *et al.*

2002). Photoperiodic modulation of leptin-induced body weight (or fat) loss is seen in the Siberian hamster, with sensitivity to leptin in two separate studies found to be greatest in a short- as opposed to a long-day photoperiod (Atcha *et al.* 2000, Klingenspor *et al.* 2000). In seasonal species like Siberian hamsters and sheep, endogenous leptin concentrations increase in long days when appetite, body weight and adiposity increase, and decrease in short days when appetite, body weight and adiposity decrease (Atcha *et al.* 2000, Klingenspor *et al.* 2000, Marie *et al.* 2001). It is postulated therefore that a transient period of relative leptin insensitivity in long days is necessary to prevent the elevated leptin levels counteracting the environmental adaptation of photoperiod-driven increases in intake and body weight in these species.

The apparent seasonal change in sensitivity of the appetite axis to leptin in the present study may have been attributable to differences in effective energy balance between the sheep at the different times of year. Appetite drive is higher in the spring (longer days) than in the autumn (shorter days), as suggested by the non-significant trend towards higher *ad libitum* intake on control treatments measured in Experiment 2 than in Experiment 1 (Fig. 2c), and live weight would normally increase in the spring as a consequence (Adam 2000). Thus the chronic

maintenance level of food intake would have imposed an effectively greater restraint on appetite and live weight in the spring, and indeed it has been reported that i.c.v. leptin does not affect appetite in food-restricted sheep with low live weight (Henry *et al.* 2001, Morrison *et al.* 2001). The ability of exogenous leptin to inhibit appetite may simply be a function of the strength of the intrinsic appetite drive that it acts against, with consequent dose dependency. It is pertinent in this regard that in the present study we found a 'pharmacological' dose-response in autumn, with only the two higher leptin doses having an effect on appetite (Experiment 1), whilst one of the same 'effective' doses of leptin had no effect on appetite in spring (Experiment 2). Our 'physiological' leptin i.c.v. infusion (Experiment 3) also had no effect on intake in spring, reflecting the seasonal insensitivity across the dose range and in support of the positive dose-response relationship. Others have reported metabolic effects of i.c.v. leptin to be dose dependent; for example, a low dose suppressed weight gain by 15% without changing daily food intake in female rats whereas a twofold higher dose decreased body weight by 30% along with a reduction in daily food intake (Dhillon *et al.* 2001).

The foregoing discussion leads to the suggestion that there may be separate sub-populations of leptin target neurones influencing the appetite and GnRH axes. Additional support for this notion comes from the present responses to murine leptin, which appeared to have similar i.c.v. biological activity to the homologous peptide with respect to its ability to decrease LH pulse amplitude and to decrease appetite by 32% (albeit non-significantly) whereas, unlike ovine leptin, it failed to affect LH pulsatility (Experiment 1; Fig. 1). This suggests that variability can exist in leptin interactions with its receptor, leading to differential responses, in this case due to a small difference in the leptin amino acid sequence (84% identical; Dyer *et al.* 1997).

In conclusion, i.c.v. leptin can stimulate LH secretion in adequately nourished sheep, with or without simultaneously inhibiting appetite. Both the appetite and LH responses are seasonally and dose dependent, yet the seasonal effect is distinct for each of these neuroendocrine axes. These results are consistent with distinct neural pathways mediating the effects of leptin on energy balance and on the reproductive neuroendocrine axis.

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