Negative energy balance and leptin regulate neuromedin-U expression in the rat pars tuberalis

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Abstract

Central neuromedin U (NMU) functions in energy balance, the hypothalamic–pituitary–adrenal axis, LH release and circadian rhythmicity. In rats, high levels of NMU occur in the hypothalamic suprachiasmatic nuclei and the pars tuberalis of the pituitary. NMU expression in the pars tuberalis appears to be downregulated in the Zucker fatty (*fa/fa*) rat, lacking functional leptin receptors. In contrast, in the dorsomedial (DMH) nuclei of the mouse, NMU expression is higher in the *ob/ob* mouse, lacking leptin, and is upregulated by fasting. However, leptin appears not to change NMU gene expression in either the mouse DMH or the rat pars tuberalis. Thus, the present study aims to better identify factors influencing central NMU expression in the rat pars tuberalis. Sprague–Dawley rats were fasted and/or challenged with intracerebroventricular leptin or ghrelin and gene expression was

measured using real-time reverse transcriptase-PCR and quantitative *in situ* hybridisation with riboprobes specific for NMU and NMU receptor (NMU-R2). NMU expression in the rat pars tuberalis was elevated by fasting. Ghrelin administration had no effect on the level of NMU expression, but leptin was found to diminish the expression in a concentration- and time-dependent manner. NMU-R2 expression was unchanged in any of the groups measured. These results suggest that NMU expression in rat pars tuberalis is upregulated in states of negative energy balance, and this may be mediated indirectly by changes in leptin levels. These results demonstrate a link between energy balance and NMU expression in the pars tuberalis of the pituitary.

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Introduction

Neuromedin U (NMU) was first isolated from porcine spinal cord and named for its potent contractile activity on the uterus. It is a highly conserved molecule existing in two molecular forms: an octapeptide (NMU-8) constituting the core active C terminus and complete 25 amino acid peptide, NMU-25 (Minamino *et al.* 1985). In the mouse central nervous system, NMU is expressed in the suprachiasmatic (SCN), dorsomedial (DMH), ventromedial (VMH) and arcuate nuclei of the hypothalamus (Graham *et al.* 2003). These nuclei are important in controlling circadian rhythms, appetite and energy balance (Takahashi & Zatz 1982, Williams *et al.* 2001). In contrast, NMU expression in the rat is limited to the SCN, relatively few diffuse cells in the arcuate nuclei and DMH in the hypothalamus and the nuclei of the solitary tract (NTS) and the inferior olive in the brainstem (Ivanov *et al.* 2002, Graham *et al.* 2003). However, relatively high levels of NMU are found in the pars tuberalis of the pituitary, which adheres closely to the ventral edge of the hypothalamic median eminence (Ivanov *et al.* 2002, Graham *et al.* 2003). Specific central receptors for NMU

(NMU-R2) are highly localised to the hypothalamic paraventricular nucleus (PVN), the ependymal lining of the hypothalamic third ventricle and the hippocampus (Howard *et al.* 2000, Guan *et al.* 2001, Graham *et al.* 2003). Central administration of NMU stimulates the hypothalamo–pituitary–adrenal axis (Hanada *et al.* 2001, Wren *et al.* 2002, Thompson *et al.* 2004), suppresses food intake and body weight gain, increases gross locomotor activity, body temperature and heat production (Howard *et al.* 2000, Nakazato *et al.* 2000). It is also involved in the regulation of circadian rhythmicity (Nakahara *et al.* 2004) and luteinizing hormone secretion (Quan *et al.* 2003). The NMU knockout mouse is hyperphagic and obese (Hanada *et al.* 2004), while the transgenic mouse, overexpressing NMU, is lean and hypophagic (Kowalski *et al.* 2005) providing strong support for the role of NMU in energy balance.

The circulating hormones, leptin and ghrelin act in opposition to one another to alter the hypothalamic gene expression of peptides involved in energy balance to maintain body weight homeostasis (Zigman & Elmqvist 2003). Leptin is secreted by, and in proportion to, the white adipose tissue and signals the status of energy stores to the hypothalamus,

inhibits feeding and increases energy expenditure (Elias *et al.* 1999). Leptin levels are lowered by fasting and food restriction (Ahren *et al.* 1997, Keim *et al.* 1998). Circulating levels of ghrelin, secreted by the stomach, rise rapidly in response to fasting and drop rapidly after feeding, and act as a short-term signal of nutrient depletion (Lee *et al.* 2002) stimulating feeding and decreasing energy expenditure via ghrelin receptors (GHS-R) in the arcuate nuclei and VMH (Kamegai *et al.* 2001, Cowley *et al.* 2003). Ghrelin levels are raised during fasting and decreased in obesity (Tschöp *et al.* 2001, Kim *et al.* 2003).

Influence of the peripheral hormones, leptin and ghrelin, on central NMU expression is at present unclear. Leptin treatment of NMU knockout mice reduces their body weight to a similar extent as wild-type mice (Hanada *et al.* 2004), indicating that the NMU system is not necessary for the central effects of leptin on appetite and body weight, despite this, NMU has been shown to partially mediate the effects of leptin on food intake in the rat (Jethwa *et al.* 2005). The level of NMU expression in the DMH of the genetically obese, leptin-deficient, *ob/ob* mouse, is higher than in wild-type littermates and NMU gene expression in this region is also elevated in response to fasting (Graham *et al.* 2003), a state in which leptin levels are lowered. These findings suggest a potential link between circulating leptin levels and regulation of central NMU gene expression. However, peripheral administration of leptin failed to change NMU gene expression in these mice (Graham *et al.* 2003). In contrast to the findings in DMH, NMU gene expression was found to be lower in the SCN of *ob/ob* mice compared to lean littermates (Howard *et al.* 2000). The level of NMU gene expression in the SCN of normal mice has also been found to show a strong circadian rhythmicity (Graham *et al.* 2005). In the Zucker fatty rat (*fa/fa*), which lacks a functional leptin receptor, the level of NMU gene expression in the pars tuberalis and pars distalis of the pituitary and the NTS is lower than in the Zucker lean animal (*fa/+*), and fasting was reported to diminish the number of cells expressing NMU in the nuclei of the solitary tract and NMU gene expression in the pars distalis (Ivanov *et al.* 2002) and VMH (Howard *et al.* 2000), implicating leptin in the control of NMU expression in the brainstem hypothalamus and pituitary of the rat. However, in another study, leptin administration has been reported to have no effect on the level of gene expression in the pars tuberalis of the rat, which was erroneously identified as the arcuate nuclei (Hanada *et al.* 2004). This indicates that the effect of fasting on NMU gene expression may be mediated via factors other than leptin.

Circulating levels of ghrelin are known to rise during fasting, in contrast to the drop in circulating leptin levels, and could potentially influence NMU gene expression. The effects of ghrelin on NMU expression have not been reported. Thus, in order to clarify the effects of fasting and the roles of both leptin and ghrelin in the regulation of NMU gene expression in the rat mediobasal hypothalamus and pars tuberalis of the pituitary gland, we have measured NMU gene

expression in Sprague–Dawley rats in response to fasting and to i.c.v.-administered leptin and ghrelin, first using real-time reverse transcriptase (RT)-PCR and secondly using the more sensitive and tissue-precise technique of quantitative *in situ* hybridisation. Gene expression of NMU-R2 in the PVN was also measured.

Material and Methods

Experimental animals

Male Sprague–Dawley rats between 8 and 10 weeks of age were obtained from Harlan Ibérica (Barcelona, Spain) and housed at the University of Santiago de Compostela, where all experimental procedures were performed. The animals were maintained in air-conditioned rooms (22–24 °C) under a controlled 14 h light:14 h darkness cycle and fed *ad libitum* with standard rat chow and water, unless otherwise indicated. Animals were killed by decapitation in a room separate from other experimental animals. Brains were removed rapidly, frozen and stored at –80 °C until cryosectioned and processed for *in situ* hybridisation. Serial sections from these animals were also used as part of a related study looking at the interactions between leptin and ghrelin on GHS-R expression (Nogueiras *et al.* 2004), thus supporting the ethos of reduction of animal numbers in experimentation. All animal experimental procedures were conducted according to the regulations of Santiago de Compostela Medical School Animal Care Research Committee.

Implantation of i.c.v. cannulas

Animals were anaesthetised by an i.p. injection of a mixture of ketamine/xylazine (ketamine 100 mg/kg BW + xylazine 15 mg/kg BW). Chronic i.c.v. cannulas were implanted stereotactically using the following coordinates: 1.3 mm posterior to bregma, 1.9 mm lateral to the midsagittal suture and to a depth of 3.5 mm as described previously (Seoane *et al.* 2003). The location of the cannula in the lateral ventricle was confirmed by methylene blue staining. Animals were caged individually and allowed to recover for a week prior to experiment. During the post-operative recovery period, the rats were handled regularly under non-stressful conditions.

Short-term ghrelin and leptin challenge

At the start of the experiment, one group of rats continued to be fed *ad libitum* and the other group was deprived of food for 48 h. Rats then received either a single i.c.v. injection of ghrelin (Bachem, Bubendorf, Switzerland; 5 µg/rat dissolved in 5 µl distilled water saturated with argon; *n*=6) or vehicle (control rats; *n*=6). Rats were killed 2 h after injection. In a separate experiment, rats were fed *ad libitum* and received a single i.c.v. injection of either ghrelin or vehicle and were killed either 1 (*n*=6) or 2 h (*n*=6) after the injection.

All treatments started at 0900 h and were carried out in the light-phase. In a separate experiment, animals were either fed *ad libitum* or fasted for 48 h as described above. A single i.c.v. injection of 5 µl containing 10 µg/rat of recombinant human leptin (Sigma) ($n=6$) or carrier ($n=6$) was given to the animals and were killed 2 h later. The doses of both ghrelin and leptin and the time of sampling after injection were decided on the basis of previous experiments, where measurable changes in gene expression have been documented at these doses and times after administration (Lopez *et al.* 2000, Seoane *et al.* 2003).

Long-term leptin challenge

Brain-infusion cannulas were stereotactically placed into the lateral ventricle as described above. A catheter tube was connected from the brain-infusion cannula to the osmotic minipump flow moderator (model 2ML2; Alza Corp., Palo Alto, CA, USA). An s.c. pocket on the dorsal surface was created using blunt dissection and the osmotic minipump was inserted. The incision was closed with sutures, and the rats were kept warm until completely recovered. Rats were then infused with either recombinant human leptin (15 µg/day) or carrier ($n=12$) for 24 ($n=6$), 48 h ($n=6$) and 7 days ($n=12$) into the lateral ventricle. For the last 48 h of the 7-day infusion period, rats were fed either *ad libitum* or fasted ($n=6$ leptin and $n=6$ carrier in both groups). During this time, rats were kept in grid-bottomed cages to facilitate food intake measurements and weighed daily. In a separate experiment, rats were infused with leptin (1 µg/day; $n=6$) or (5 µg/day; $n=6$) for 7 days as described above.

RNA extraction and real-time RT-PCR

Total RNA was extracted by Trizol reagent (Invitrogen). Rat hypothalamic expression of the mRNA-encoding NMU was assessed by real-time RT-PCR. Total RNA (2 µg) was reverse transcribed using random primer and Superscript III reverse transcriptase (Invitrogen). Real-time RT-PCR analyses were performed in the ABI 7700 sequence detection system (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). Reactions were performed in duplicate. The synthesised cDNAs were further amplified by PCR using SYBR Green I as fluorescent dye and 1×PCR Master Mix (Applied Biosystems) containing 300 nmol/l of forward and reverse primers, in a final volume of 25 µl. All reactions were carried out using the following cycling parameters: 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Product purity was confirmed by dissociation curves and random agarose gel electrophoresis. No template controls were included in all the assays, yielding no consistent amplification. Standard curves were constructed for each specific target and the internal control by plotting values of CT (the cycle at which the fluorescence signal exceeds background) versus log cDNA input (in nanograms). Accordingly, CT values from each experimental sample were

then used to calculate the amount of each gene and cyclophilin A mRNAs relative to the standard. For each sample, results in terms of gene expression levels were normalised to those of the internal control, cyclophilin A.

The oligonucleotide-specific primers for rat NMU and cyclophilin A are as follows: NMU forward 5'-TGC GTC CTT TCT GTC TGT TG-3'; reverse 5'-TCA TGC AGT TGA GGA ACG AG-3'; cyclophilin A forward 5'-AGC ACT GGG GAG AAA GGA TT-3'; reverse 5'-CAT GCC TTC TTT CAC CTT CC-3'.

In situ hybridisation

The mRNA levels for rat NMU, Ob-Rb and NMU-R2 were quantified using specific probes derived as previously described (Graham *et al.* 2003) using quantitative *in situ* hybridisation (Mitchell *et al.* 2002). Briefly, 20 µm thick, coronal hypothalamic section was fixed in 4% (w/v) paraformaldehyde in 0.1 mol/l PBS for 20 min at room temperature, washed in PBS, incubated in 0.1 mmol/l triethanolamine for 2 min and acetylated in 0.1 mmol/l triethanolamine and 0.25% (v/v) acetic anhydride for 10 min. Sections were dehydrated through a graded series of ethanol and dried under vacuum before hybridisation with riboprobes at 10^6 c.p.m./ml for 18 h at 58 °C. After hybridisation, sections were desalted through a series of washes in SSC to a final stringency of $0.1 \times \text{SSC}$ at 60 °C for 30 min, treated with RNase A and dehydrated in ethanol. Air-dried slides were apposed to Biomax MR (Sigma) together with autoradiographic (^{14}C) microscale standards (Amersham) for 1 week at room temperature, for autoradiography. Slides were then dipped in Hypercoat LM-1 Nuclear Emulsion (Amersham) and exposed for approximately 1 month for light microscopy. Images were captured on a Leica DMR microscope equipped with Hamamatsu C5810-10 3CCD colour camera using Image Pro Plus software (Media Cybernetics, Berks, UK).

Quantification of in situ hybridisation

Autoradiographs of sections and (^{14}C) microscale standards were scanned on Umax Power Look II (UMAX Data Systems, Fremont, CA, USA). Integrated optical densities of pars tuberalis and PVN were measured using the Image Pro-Plus system (Media Cybernetics) and converted to nCi/g using a standard curve generated from the (^{14}C) microscales. Values were then converted to percentages of control values (100%) for each separate *in situ* hybridisation experiment in order to combine the data.

Statistical analysis

Data were represented as means $\pm$ S.E.M. and analysed (Fig. 2–5) by two-way ANOVA followed by Student–Newman–Keuls method. For experiments where the effect of leptin vs saline infusion were compared on body weight and food intake and where the time and dose dependency of leptin treatment were

measured (Fig. 6) data were analysed by one-way ANOVA. A value of $P < 0.05$ was considered statistically significant.

Results

Localisation of NMU and Ob-Rb gene expression

NMU gene expression is clearly localised to the pars tuberalis of the pituitary (Fig. 1A). There is no apparent overlap

between NMU gene expression and Ob-Rb gene expression, which is localised to several nuclei in the mediobasal hypothalamus with no apparent expression over the pars tuberalis (Fig. 1B and C). Dark-field microscopy of NMU gene expression on emulsion-coated slides shows a thin layer of silver grains (Fig. 1D) corresponding to the pars tuberalis at the outer edge of the median eminence (Fig. 1E and F). A number of relatively diffuse and sparsely labelled NMU-expressing neurons were seen in the arcuate and VMH as

Figure 1 (A) NMU mRNA expression in the rat brain. Specific signal is clearly localised to the pars tuberalis at the ventral edge of the median eminence. (B) Ob-Rb mRNA expression in the mediobasal hypothalamus, the area of the median eminence and pars tuberalis is devoid of signal. (C) Stained section that gave rise to image in (B), showing the location of the pars tuberalis. (D) Dark-field image of NMU gene expression in the rat pars tuberalis. Silver grains appear white. (E) Corresponding bright-field image of the section underlying the dark-field image. (F) High magnification of bright-field image of NMU gene expression. Silver grains can clearly be seen overlying the pars tuberalis tissue.

previously reported (not shown). However, the scarcity of these neurones in the sections observed in the present study made it impossible to carry out quantification of NMU gene expression by quantitative *in situ* hybridisation in the mediobasal hypothalamus. The NMU probe used in the present study has a portion of overlap with the known gene sequence of neuromedin S (NMS) and the possibility exists that some of the signal located in the present study corresponds to NMS gene expression. However, this is unlikely as NMS is almost exclusively localised to the SCN and its occurrence in the pars tuberalis has not been reported despite sections containing the closely neighbouring arcuate nuclei being observed (Mori *et al.* 2005).

Long-term leptin challenge in fed and fasted rats by real-time RT-PCR

Two-way ANOVA, to test the effect of treatments and/or interactions between treatments, revealed that there was a significant effect ($P < 0.01$) of fasting to increase the expression of NMU in the dissected area, while the overall effect of leptin was not significant ($P = 0.058$). There was no interaction between leptin and fasting as factors influencing NMU gene expression (Fig. 2). However, as the statistical significance for the effect of leptin was judged as borderline and the variability of the results was large as witnessed by the size of the standard errors, it was decided that the use of a more sensitive and region-specific technique, such as quantitative *in situ* hybridisation may be able to detect pars

Figure 2 (A) Using real-time RT-PCR, fasting for 48 h was found to significantly increase NMU gene expression ($P < 0.01$), while leptin infusion (15 µg/day) for 1 week had no overall effect on NMU expression in the pars tuberalis ($P = 0.058$) by two-way ANOVA. Student–Newman–Keuls analysis revealed significant differences between fed vs fasted vehicle-challenged animals, and fed, leptin-challenged vs fasted, vehicle-challenged, and fasted vehicle-challenged vs fasted leptin-challenged groups. Differences between individual groups are indicated by * $P < 0.05$.

tuberalis-specific changes in response to leptin treatment. Student–Newman–Keuls analysis revealed a significant difference ($P < 0.05$) between fed groups versus fasted vehicle-treated group, and fasted vehicle-treated and fasted leptin-treated groups.

Short-term leptin challenge in fed and fasted rats by quantitative *in situ* hybridisation

Two-way ANOVA revealed that there was an effect of 48 h fasting on the level of NMU gene expression in the pars tuberalis when compared to rats fed *ad libitum* ($P < 0.01$); fasting increased NMU gene expression (Fig. 3A). There was also an effect of i.c.v. leptin treatment on the level of NMU expression in the pars tuberalis; leptin reduced NMU expression ($P < 0.05$). There was no interaction between fasting and leptin as factors influencing NMU gene expression. Student–Newman–Keuls analysis revealed a single significant difference ($P < 0.05$) between the fed leptin-treated group and the fasted control group. NMU-R2 mRNA expression was not influenced by either fasting or i.c.v. leptin injection (Fig. 3B).

Short-term ghrelin challenge in fed and fasted rats by quantitative *in situ* hybridisation

Two-way ANOVA revealed that there was an effect of fasting to increase NMU gene expression in the pars tuberalis ($P < 0.001$). Ghrelin, given i.c.v., had no significant effect on NMU mRNA expression in the pars tuberalis either 1 (not shown) or 2 h after injection in rats fed *ad libitum* or 48 h fasted (Fig. 4A). Student–Newman–Keuls analysis revealed a significant difference ($P < 0.05$) between the fed and the fasted groups. No significant effect of fasting or ghrelin treatment (1 or 2 h) was observed on NMU-R2 gene expression in the PVN (Fig. 4B).

Long-term leptin challenge in fed and fasted rats by quantitative *in situ* hybridisation

Long-term i.c.v. leptin infusion (15 µg/day) significantly decreased food intake and body weight, as described earlier (Nogueiras *et al.* 2004). The final body weight of leptin-treated rats was 310 ± 7 g compared to 350 ± 5 g ($P < 0.001$) for the saline infused animals. Daily food intake for the leptin-infused animals was 19 ± 0.9 g compared to 27 ± 0.5 g for the saline-infused animals ($P < 0.001$). Two-way ANOVA revealed that the leptin-treated group had significantly lower NMU mRNA levels in the pars tuberalis compared to vehicle-treated animals ($P < 0.001$), and that fasting increased NMU gene expression ($P < 0.05$). Student–Newman–Keuls analysis revealed significant differences ($P < 0.05$) between all groups (Fig. 5A). However, long-term leptin administration had no significant effect on NMU-R2 mRNA levels in either fed or fasted rats (Fig. 5B).

Figure 3 (A) Using quantitative *in situ* hybridisation both fasting for 48 h and i.c.v. leptin 2 h after injection had significant effects on NMU gene expression ($P < 0.01$, < 0.05) in the pars tuberalis by two-way ANOVA. Student–Newman–Keuls analysis revealed that there was a significant difference between rats fed *ad libitum*, leptin-injected and 48 h fasted, $*P < 0.05$. (B) NMU-R2 gene expression in the PVN, where no significant difference was detected. Representative autoradiographic images underlie the histograms.

Leptin time and dose response

Leptin i.c.v. infusion of either 1 or 5 $\mu\text{g}/\text{day}$ for 1 week, significantly ($P < 0.01$ and < 0.001 respectively) decreased the level of NMU expression. The level of NMU expression was significantly lower ($P < 0.01$) after infusion of 5 $\mu\text{g}/\text{day}$ leptin than after 1 $\mu\text{g}/\text{day}$ leptin (Fig. 6A). Leptin i.c.v. infusion of 15 $\mu\text{g}/\text{day}$, both for 24 ($P < 0.01$) or 48 h ($P < 0.001$), significantly decreased NMU gene expression in the rat pars tuberalis compared to control animals. The level of NMU gene expression at 48 h was significantly lower than that at 24 h ($P < 0.05$; Fig. 6B).

Figure 4 (A) Using quantitative *in situ* hybridisation there was no effect of i.c.v. ghrelin 2 h after injection on NMU gene expression in the pars tuberalis, whereas fasting for 48 h had a significant effect on NMU gene expression ($P < 0.001$) by two-way ANOVA. Student–Newman–Keuls analysis revealed that there was a significant difference between all fed and fasted groups irrespective of ghrelin treatment. Representative autoradiographic images underlie the histograms. (B) NMU-R2 gene expression in the PVN, where no significant difference was detected.

Discussion

The present study shows that NMU gene expression on the rat pars tuberalis is increased in response to fasting and decreased in a dose- and time-dependent manner by central leptin administration. Central ghrelin administration at the times and doses tested had no significant effect on NMU gene expression. These results indicate that NMU gene expression in the pars tuberalis is responsive to nutritional status and leptin. These results also emphasise the importance of using a highly sensitive and region-specific technique in the analysis of homogeneous tissues such as the hypothalamus and the adjoining pars tuberalis. The function of NMU in the pars tuberalis is at present unclear. The pars tuberalis is thought to function as a timer of seasonal physiology,

Figure 5 (A) Using quantitative *in situ* hybridisation, leptin infusion (15 µg/day) for 1 week was found to decrease NMU expression in the pars tuberalis ($P < 0.01$), while fasting for 48 h significantly increased gene expression ($P < 0.01$) by two-way ANOVA. Student–Newman–Keuls analysis revealed significant differences between fed vs fasted vehicle-challenged animals, and fed, vehicle-challenged vs fed leptin-challenged, and fasted vehicle-challenged vs fasted leptin-challenged. Differences between individual groups are indicated by * $P < 0.05$. Representative autoradiographic images underlie the histograms. (B) NMU-R2 gene expression in the PVN, where no significant difference was detected.

integrating the melatonin signal with endogenous cycles of clock genes expressed in this region of the pituitary. As such, the pars tuberalis is influential in controlling the annual cycles of reproduction, pelage growth and moulting and appetite and body weight in seasonal animals (Lincoln *et al.* 2003). The laboratory rat maintains highly dampened seasonal cycles in reproduction and energy balance, which appear to be strain-dependent, but may be unmasked by food restriction (Francisco *et al.* 2004).

The role of central NMU in food intake and energy balance is complex. In the rat, while a single i.c.v. injection of NMU gives a clear catabolic response, increasing core body temperature and locomotor activity, reducing food intake (Nakazato *et al.* 2000) and acute administration of NMU into the PVN has clear anorexigenic effects (Wren *et al.* 2002);

Figure 6 (A) Effect of leptin on NMU mRNA expression in the pars tuberalis after 1 week of infusion measured by quantitative *in situ* hybridisation, † $P < 0.01$ leptin 1 µg/day vs vehicle, ‡ $P < 0.001$ leptin 5 µg/day vs vehicle, † $P < 0.01$ 1 µg/day vs 5 µg/day by one-way ANOVA. (B) Effect of leptin (15 µg/day) on NMU mRNA expression in the pars tuberalis; † $P < 0.01$ 24 h leptin vs vehicle and ‡ $P < 0.001$ 48 h leptin vs vehicle and * $P < 0.05$ 24 h leptin vs 48 h leptin by one-way ANOVA. Representative autoradiographic images of NMU gene expression in the pars tuberalis from treatments underlie the histograms.

however, chronic injections of NMU into the PVN have no effect on food intake or body weight. Nevertheless, the NMU knockout mouse shows clear hyperphagia and obesity (Hanada *et al.* 2004) and the transgenic mouse overexpressing NMU is lean and hypophagic (Kowalski *et al.* 2005). Recently, it has been demonstrated that at least some of the effects of leptin are mediated via NMU (Jethwa *et al.* 2005). It has been shown that fasting increases NMU mRNA levels in the DMH hypothalamic nuclei of mice (Graham *et al.* 2003). In addition, leptin-deficient *ob/ob* mice have been reported to have higher NMU mRNA expression in the DMH than lean littermates. In both cases, leptin levels are low or absent and the animals have a strong drive to eat, contrasting with the reported anorexigenic effects of NMU. However, NMU gene expression in the SCN has been reported to be higher in the *ob/ob* mouse compared to lean littermates (Howard *et al.* 2000) and show clear circadian changes in the level of expression (Graham *et al.* 2005),

linking energy balance and circadian clock function. In the Zucker fatty rat, lower levels of gene expression of NMU in the pars tuberalis and pars distalis of the pituitary, and in the NTS, have been reported compared to the lean animal (Ivanov *et al.* 2002), and fasting has been found to decrease NMU expression in the nuclei of the solitary tract and significantly decrease NMU gene expression in the pars distalis of the pituitary (Ivanov *et al.* 2002) and VMH (Howard *et al.* 2000). This reduced NMU gene expression appears to be linked to an absent or diminished leptin signal. In contrast, the results of the present study show clearly that fasting for 48 h upregulates NMU gene expression in the pars tuberalis. This finding, like those in the mouse detailed above, is difficult to explain in the context of NMU acting in the brain as an anorexigenic agent and may indicate that the function of NMU in the pars tuberalis is unrelated to appetite and energy balance control.

Negative energy balance induced by fasting does, however, clearly upregulate NMU gene expression on the rat pars tuberalis. The present study also clearly shows the influence of leptin on NMU expression in the rat pars tuberalis with discernable decreases in the expression of NMU in both fed and fasted rats 2 h after a single i.c.v. injection of leptin and more marked decreases after infusion of leptin for 1 week. Downregulation of gene expression can be difficult to demonstrate as the half-life of the RNA transcripts differ and downregulation of certain genes may take longer to become apparent. This may explain a contradictory report of the effect of leptin on NMU gene expression in the pars tuberalis (identified as the arcuate nuclei in that study), in which length of time after leptin administration was not documented (Hanada *et al.* 2004). In the present study, while a single i.c.v. leptin injection was shown to have an effect on NMU expression, using quantitative *in situ* hybridisation, the effect became more pronounced in rats challenged for 24 h or longer. While leptin treatment clearly downregulated NMU gene expression in both a time- and dose-dependent manner, the lack of leptin receptor (Ob-Rb) expression on the rat pars tuberalis indicates that the effect of leptin on NMU gene expression is indirect. A direct influence of leptin on NMU expression is unlikely as leptin receptors are absent from the rat pars tuberalis. However, the pars tuberalis is in close contact with the median eminence, both directly as the two tissues oppose each other and indirectly via the blood supply in the primary portal plexus (Wittkowski *et al.* 1992), meaning that any secretion from the median eminence reaches the pars tuberalis almost immediately and in relatively high concentrations. Thus, any influence of leptin on the hypothalamus could impact the output of the median eminence, which in turn could influence NMU gene expression in the pars tuberalis. When leptin levels are low, ghrelin levels tend to be high (Cummings & Foster 2003, Kim *et al.* 2003), making ghrelin another likely candidate for mediating this effect of negative energy balance on NMU expression. However, the present study found no

demonstrable effects of i.c.v. ghrelin administration on the level of NMU expression at the doses and times tested.

The downregulation of NMU gene expression in the pars tuberalis by leptin is also at odds with the known anorexigenic action of NMU and further suggests that the function of NMU in the pars tuberalis is unrelated to appetite control and energy homeostasis. As already detailed, the pars tuberalis is a region of the pituitary that is involved in seasonality and is a major site of action of melatonin (Williams 1989). The pars tuberalis also controls prolactin release from the pars distalis via an, as yet, unidentified, juxtacrine agent (Morgan *et al.* 1996, Johnston 2004). Interestingly, a novel 33-residue peptide derived from the NMU proprotein has recently been identified and shows potent prolactin-releasing activity when administered i.c.v. (Williams *et al.* 2001, Mori *et al.* 2005). In the present study, the interaction of nutritional status and leptin with NMU gene expression in the pars tuberalis is clearly evidenced. However, the function of NMU in the pars tuberalis is not known and the relevance of the relationship between nutritional status, leptin and the level of NMU gene expression in this region of the pituitary is not clear at present.

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