Diurnal changes in hypothalamic neuropeptide and SOCS-3 expression: effects of lactation and relationship with serum leptin and food intake

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Abstract

Rats normally eat about 85% of their food at night. Lactation increases food intake 3- to 4-fold, but the diurnal pattern of food intake persists. The mechanisms responsible for the diurnal and lactation-induced changes in food intake are still unresolved, hence we have further investigated the possible roles of serum leptin and hypothalamic expression of neuropeptide Y (NPY), agouti-related peptide (AgRP) and pro-opiomelanocortin (POMC) in rats. Suppressor of cytokine signalling-3 (SOCS-3) acts as a feedback inhibitor of leptin signalling in the hypothalamus, hence changes in expression of SOCS-3 were also investigated.

Changes in expression of NPY, AgRP or POMC alone could not account for the diurnal changes in intake and their alteration by lactation. However, there were increased AgRP mRNA:POMC mRNA ratios at night and also during lactation, which were very similar to

estimated changes in food intake. Such changes in expression may result in dominance of the orexigenic AgRP peptide over the appetite-suppressing POMC-derived peptides, and so could contribute to the hyperphagia in these states. Diurnal and lactation-related changes in the AgRP mRNA:POMC mRNA ratio and food intake are not due to changes in leptin alone. However, hypoleptinaemia, possibly through increased expression of NPY, may contribute to the hyperphagia of lactation.

In the dark, expression of SOCS-3 was decreased in non-lactating rats; lactation decreased SOCS-3 expression in both light and dark phases. However, such changes are likely to enhance the ability of leptin-responsive neurones to transmit the leptin signal, and so are unlikely to contribute to either the nocturnal increase in appetite or the hyperphagia of lactation.

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Introduction

Rats are primarily nocturnal and eat about 85% of their food at night (Kimura *et al.* 1970, Bruckdorfer *et al.* 1974). Lactation increases food intake 3- to 4-fold in rats, reflecting the considerable nutritional demands of the mammary gland (Wade & Schneider 1992, Barber *et al.* 1997), but the diurnal pattern of food intake persists, with about 65% of food still being consumed at night (Strubbe & Gorissen 1980, Munday & Williamson 1983, Pickavance *et al.* 1996, Denis *et al.* 2003a). Despite the hyperphagia, lactating rats enter and remain in a state of overall net negative energy balance (Barber *et al.* 1997). The mechanisms regulating both the diurnal changes in food intake and the hyperphagia of lactation are not resolved.

Central control of appetite involves numerous neural circuits, including the hypothalamic neurones expressing

neuropeptide Y (NPY) and agouti-related peptide (AgRP), and others that produce pro-opiomelanocortin (POMC), the precursor of α -melanocyte-stimulating hormone (α -MSH; Elmquist *et al.* 1999, Schwartz *et al.* 2000, Williams *et al.* 2001, Pritchard *et al.* 2002). NPY and AgRP stimulate, while α -MSH inhibits feeding. α -MSH activates the melanocortin-4 receptor (MC4-R), which is antagonised by AgRP (Elmquist *et al.* 1999, Schwartz *et al.* 2000, Williams *et al.* 2001).

Leptin, a peptide hormone secreted predominantly by adipocytes, acts on the hypothalamus to restrain appetite and increase energy expenditure (Friedman & Halaas 1998, Ahima & Flier 2000, Ahima *et al.* 2000, Elmquist 2001). These central actions are partly explained by leptin's ability to inhibit the NPY/AgRP neurones while stimulating the POMC neurones (Ahima & Flier 2000, Ahima *et al.* 2000, Schwartz *et al.* 2000, Elmquist 2001,

Williams *et al.* 2001). During periods of negative energy balance, serum leptin concentrations fall, leading to increased expression of NPY and AgRP, and decreased expression of POMC in the hypothalamus; these changes are probably important in increasing hunger and decreasing energy expenditure in conditions such as fasting and diabetes (Friedman & Halaas 1998, Ahima 2000, Ahima & Flier 2000, Ahima *et al.* 2000, Williams *et al.* 2001).

Leptin apparently modulates its own effects within the hypothalamus, as it inhibits the expression of the 'b' isoform of the leptin receptor (Ob-Rb) and induces down-regulation of the receptor protein (Mercer *et al.* 1997, Baskin *et al.* 1998, Uotani *et al.* 1999). Leptin also induces expression of suppressor of cytokine signalling-3 (SOCS-3), a protein that inhibits a critical intracellular signalling pathway activated by leptin (Björbaek *et al.* 1999).

The mechanisms driving the diurnal pattern of feeding in rats are unexplained. Serum leptin concentrations are increased at night (Pickavance *et al.* 1998, Xu *et al.* 1999, Ahren 2000, Pu *et al.* 2000, Kalsbeek *et al.* 2001). Thus, the diurnal pattern of feeding in rats is not due to changes in serum leptin concentration; rather, the nocturnal rise in serum leptin is at least partly due to the increase in food intake (Ahima *et al.* 1998, Xu *et al.* 1999). Hypothalamic NPY expression is decreased at night in male rats (Xu *et al.* 1999), which is consistent with the nocturnal rise in serum leptin, but not with increased food intake. By contrast, AgRP expression increases at night in male rats (Lu *et al.* 2002); this could contribute to nocturnal hyperphagia, but appears inconsistent with the hyperleptinaemia. Reports of diurnal changes in POMC expression are inconsistent (Wise *et al.* 1990, Steiner *et al.* 1994, Xu *et al.* 1999, Lu *et al.* 2002).

The nocturnal increase in serum leptin is markedly attenuated and daytime leptin concentration appears generally decreased during lactation (Johnstone & Higuchi 2001, Smith & Grove 2002, Vernon *et al.* 2002); this hypoleptinaemia is thought to facilitate the hyperphagia of lactation. Consistent with the general decrease in serum leptin, hypothalamic expression of NPY and AgRP are increased during the daytime in lactating rats, while POMC is decreased (Smith & Grove 2002, Vernon *et al.* 2002). The effects of lactation on the expression of these hypothalamic neuropeptides at night is not known.

Here, we investigated further the mechanisms responsible for the diurnal pattern of food intake in rats – and its modulation by lactation – by determining diurnal changes in hypothalamic neuropeptide and SOCS-3 expression, their relationships to serum leptin and their modification by lactation. Changes in SOCS-3 expression do not explain the nocturnal or lactation-induced hyperphagia. However, we suggest that changes in the ratio of AgRP:POMC expression, and hence signalling through the MC4-R system, are important both for the diurnal rhythm of food intake and for the hyperphagia of lactation. Furthermore, neither changes in the

AgRP:POMC expression ratio nor food intake can be attributed to changes in leptin alone.

Materials and Methods

Animals

Female Wistar rats, raised at the Hannah Research Institute from stock obtained originally from A. Tuck and Sons, Ltd (Rayleigh, Essex, UK), were fed standard pelleted chow *ad libitum* (CRM diet, Labsure, Poole, Dorset, UK) and had free access to water. They were divided into two groups of 16 animals: one was maintained on a light–darkness schedule with lights on from 0700 h to 1900 h and the other with lights on from 2200 h to 1000 h. Eight rats from each group were mated at about 10 weeks of age, while the others were kept for non-lactating controls. Litter size was adjusted to 10 pups within 24 h of birth. All animal procedures were approved by the Ethical Review Committee of the Hannah Research Institute.

After at least 4 weeks exposure to the specific light–darkness regimen, non-lactating and lactating rats (day 14 of lactation) were killed 2 h into the light or dark phase. Trunk blood was collected and allowed to clot, and serum stored at -20°C for later analyses. The brain was frozen in liquid nitrogen and the hypothalamus dissected out *en bloc* under a binocular microscope, using consistent landmarks (Williams *et al.* 1988). The hypothalamic block was removed from a frontal slice of brain tissue between the optic chiasm and the mammillary bodies, using cuts made immediately below the anterior commissure and through the perihypothalamic sulci. The block was kept frozen in liquid nitrogen until mRNA extraction.

Serum leptin

Serum leptin concentration was determined by RIA using a commercial kit (Linco Research, Inc. MO, USA; distributed by Biogenesis, Poole, Dorset, UK).

Quantitative reverse transcription (RT)-PCR

RT-PCR was used to measure NPY, AgRP, POMC and SOCS-3 mRNAs. Total RNA was extracted from frozen dissected hypothalami, using Tri Reagent (Sigma). The yield and quality of extracted RNA were assessed by the 260:280 nm optical density ratio and electrophoresis, under denaturing conditions, on 1.1% agarose gel. Only samples displaying satisfactory quality were used for analysis. Total RNA was subjected to treatment with RNase-free DNase I (Roche) for 10 min at 37°C and a further 10 min at 75°C .

Reverse transcription was carried out using optimized amounts (1 μg) of total RNA with a Reverse-iT 1st Strand synthesis kit (Abgene, Epsom, Surrey, UK) in a final

volume of 25 µl. First-strand cDNA species were PCR amplified using the following primers, obtained from Sigma-Genosys Ltd (Pampisford, UK) (sequence alignments were performed with the BLAST system): NPY, 5'-C GCCATGATGCTAGGTAAC-3' and 5'-CAGA CTGGTTTCACAGGATG-3' for a 379 bp sequence (Rybkin *et al.* 1997, accession number M20373); AgRP, 5'-AGAGTTCTCAGGTCTAAGTCT-3' and 5'-CTT GAAGAAGCGG CAGTAGCAC-3' corresponding to a 321 bp sequence (Korner *et al.* 2000, AF206017); POMC, 5'-AGCCTCTGTCCAGTCCTGAG-3' and 5'-CTTA GTCAGTCTCCTTAAC-3' corresponding to a 349 bp sequence (Drouin *et al.* 1985, K01877); SOCS-3, 5'-AC CAGCGCACTTCTTCACA-3' and 5'-GTGGAG CATCATACTGGTCC-3' corresponding to a 450 bp sequence (Björbaek *et al.* 2001, AJ249240). Complementary DNA was also generated for the housekeeping gene hypoxanthine-guanine phospho-ribosyltransferase (HPRT; Siegling *et al.* 1994, X62085) using 5'-CAG TCCCAGCGTCGTGATTA-3' and 5'-AGCAAGTC TTTCAAGTCCTGTC-3' corresponding to a 138 bp sequence.

For amplification of cDNA, PCR was performed with 1 µl template cDNA and 35 µl PCR mix (1.1 × ReddyMix PCR master mix, 1.5 mM MgCl₂, ABgene) on a thermal cycler (Hybaid, Ashford, UK). After initial denaturation at 94 °C for 5 min, the samples were subjected to cycles of amplification: denaturation at 94 °C for 30 s; annealing for 30 s at 58 °C for AgRP, POMC and SOCS-3, and 56 °C for HPRT and NPY; and extension for 40 s at 72 °C. Preliminary PCR experiments showed that the rate of amplification was linear for a limited number of cycles (data not shown), so the samples were subjected to 29 cycles of amplification for HPRT, 32 cycles for NPY and AgRP, and 38 cycles for SOCS-3 and POMC. As competition was observed between HPRT and other primers, each cDNA was amplified in individual tubes. After final extension at 72 °C for 7 min, 15 µl of each mixture was subjected to electrophoresis on a 1.1% agarose gel. Controls without reverse transcriptase were systematically performed to detect cDNA contamination. Bands were visualized and quantified using a digital image analysis system (Kodak 1-D, Kodak Eastman). Optical density was calculated for each gene product and the results expressed as the ratio of its density to that of the HPRT product.

Statistical analyses

Results were analysed by ANOVA, with physiological state, light/dark phase and their interactions as factors. For significant state-by-phase interactions, Tukey's *post-hoc* test was used to test the difference between means. Multiple regression analysis was used to investigate possible linear relationships of neuropeptide and SOCS-3 expression to serum leptin, and of POMC to SOCS-3

expression, together with the effect of physiological state and light/dark phase on these relationships. The fitted lines shown in Figs 2 and 3 correspond to the best-fitting regression models according to tests of significance. A *P* value of 0.05 or less was considered as significant. All analyses were performed using Genstat (Genstat, 6th edition, Lawes Agricultural trust, VSN International Ltd, Oxford, UK), except for Tukey's test for which Minitab (Minitab 13, Minitab Inc., State College, PA, USA) was used.

Results

Food intake and serum leptin concentration

Daily food intake and serum leptin results from this experiment have already been described (Denis *et al.* 2003b). In brief, daily food intake was 14.3 ± 1.7 and 58.2 ± 3.8 g/rat for non-lactating and lactating rats respectively ($P < 0.001$). Serum leptin concentrations were 1.81 and 3.79 ng/ml for non-lactating rats in the light and dark respectively, and 1.55 and 2.53 ng/ml for lactating rats in the light and dark phases respectively; all values are means of eight observations, and the common S.E.M. from ANOVA was 0.22. The nocturnal increase in leptin was significant in both states ($P < 0.001$ for non-lactating and $P = 0.017$ for lactating rats), and was significantly diminished ($P = 0.026$) by lactation. Lactation had no significant effect on serum leptin concentration during the light phase.

Expression of hypothalamic genes

NPY mRNA levels were significantly increased by lactation ($P < 0.001$), but did not vary significantly with light/dark phase ($P = 0.073$) (Fig. 1A). Lactation increased ($P = 0.008$) AgRP expression during the dark phase, but not in the light (Fig. 1B). There was no effect of light/dark phase on AgRP expression in either state (Fig. 1B). In non-lactating rats POMC expression was lower ($P = 0.002$) in the dark phase than in the light (Fig. 1C). Lactation decreased expression of POMC in the light ($P = 0.002$), but did not decrease expression further in the dark (Fig. 1C). SOCS-3 expression was lower at night in both non-lactating and lactating rats ($P = 0.002$) and was decreased during lactation in both light and dark phases ($P = 0.019$) (Fig. 1D). The ratio of AgRP:POMC expression was higher at night in both physiological states ($P = 0.001$) and was increased by lactation ($P = 0.002$) in both light and dark phases (Fig. 1E).

Relationships between hypothalamic gene expression and serum leptin concentration

There were significant, negative relationships between NPY ($P = 0.037$, Fig. 2A), POMC ($P = 0.007$, Fig. 2B)

and SOCS-3 ($P=0.004$, Fig. 2C) and serum leptin. However, in each case the intercept with the y -axis, but not the gradient, was altered by state; for NPY, the intercept was also affected by light/dark phase (Fig. 2). For both POMC and SOCS-3 there was a significant effect of state ($P<0.001$ and $P=0.002$ respectively), such that for a given leptin concentration, less POMC and SOCS-3 was expressed during lactation (Fig. 2B and C). By contrast, for NPY the effect of lactation was different in the light and dark ($P=0.02$, Fig. 2A); in the light, less NPY was expressed for a given leptin concentration in non-lactating rats than during lactation ($P<0.001$, Fig. 2A), whereas there was no effect of lactation in the dark phase. No significant relationships were found between serum leptin concentration and either hypothalamic AgRP expression or AgRP:POMC ratio ($P=0.622$ and $P=0.806$ respectively) (results not shown).

There was a highly significant positive relationship between POMC and SOCS-3 expression ($P<0.001$) (Fig. 3). The gradient did not change significantly with state or light/dark phase, but the y -intercept varied, such that there was more POMC for a given amount of SOCS-3 in non-lactating rats in the light phase than in the other three situations ($P\leq 0.011$, Fig. 3).

Discussion

Diurnal changes in hypothalamic gene expression: relationship to leptin and food intake

The mechanisms regulating the diurnal changes in food intake in rats are poorly understood. Serum leptin, which is usually anorexic, is increased at night (Pickavance *et al.* 1998, Xu *et al.* 1999, Ahren 2000, Mastronardi *et al.* 2000, Nishiyama *et al.* 2000, Pu *et al.* 2000, Ziotopoulou *et al.* 2000, Kalsbeek *et al.* 2001, Denis *et al.* 2003a), probably due to the increase in food intake around the onset of night (Ahima *et al.* 1998, Xu *et al.* 1999). Whether the elevated leptin levels act to constrain nocturnal eating (that is, rats would eat even more if leptin concentration did not increase) or whether the hypothalamus becomes refractory to leptin at night is not known.

In male rats, increased AgRP expression at night suggests that it could promote nocturnal eating (Lu *et al.* 2002), whereas a decrease in NPY expression at night (Xu *et al.* 1999) argues against involvement of NPY. In contrast to these studies with male rats, we found no

diurnal change in either NPY or AgRP expression in the hypothalamus in non-lactating female rats. Our finding that POMC expression is decreased in the early hours of the dark period in non-lactating, female rats agrees with the results of Wise *et al.* (1990). Published diurnal changes in hypothalamic POMC expression in male rats are not consistent, but two reports suggest that expression may decrease at the onset of the dark period (Steiner *et al.* 1994, Xu *et al.* 1999), although no change was found by Lu *et al.* (2002). A decrease in hypothalamic POMC expression around the beginning of the dark period may thus contribute to the nocturnal hyperphagia in both sexes.

There was a negative correlation between NPY expression and serum leptin, which is consistent with known effects of leptin on hypothalamic NPY expression (Ahima & Flier 2000, Ahima *et al.* 2000, Schwartz *et al.* 2000, Elmquist 2001, Williams *et al.* 2001). By contrast, there was no apparent relationship between serum leptin and AgRP expression, while a seemingly paradoxical, negative relationship between serum leptin and hypothalamic POMC expression was apparent. The latter, however, is probably due to the decrease in POMC expression in the dark, at a time when serum leptin is increased. Whether this rise in leptin subsequently causes an increase in POMC expression further into the dark period is unclear. Leptin infusion decreased NPY expression, but had no effect on POMC expression in fed rats (Ahima *et al.* 1999) and mice (Takahashi *et al.* 2002), implying a differential sensitivity to leptin of their respective neurones. Refractoriness of POMC neurones to leptin in the fed rat could explain why food intake is increased throughout the night despite continuing high levels of leptin (Xu *et al.* 1999, Ahren 2000, Mastronardi *et al.* 2000, Ziotopoulou *et al.* 2000, Kalsbeek *et al.* 2001, Nishiyama *et al.* 2001, Denis *et al.* 2003a).

Leptin increases hypothalamic expression of SOCS-3, and this then acts as a feedback inhibitor of leptin signalling (Björbaek *et al.* 1999). SOCS-3 expression is also regulated by other hormones and cytokines (Björbaek *et al.* 1999, Auernhammer & Melmed 2001), hence we hypothesised that an increase in SOCS-3 at night could act to limit the response to leptin and so contribute to the hyperphagia. However, contrary to this idea, SOCS-3 expression fell during the dark despite the hyperleptinaemia. SOCS-3 is expressed in both POMC and NPY neurones (Elias *et al.* 1999, Baskin *et al.* 2000), but we suggest that the diurnal, and also lactation-induced,

Figure 1 Amount of NPY mRNA (A), AgRP mRNA (B), POMC mRNA (C) and SOCS-3 mRNA (D) relative to HRPT mRNA, and the ratio of AgRP:POMC mRNA (E) in the hypothalamus of non-lactating and lactating rats in the light and dark phases. Values are means $\pm$ S.E.M., obtained by ANOVA, of eight observations for non-lactating rats, and six and eight observations for lactating rats, in the light and dark phases respectively. For NPY there was a significant effect of physiological state ($P<0.001$) but not light/dark phase ($P=0.073$). For SOCS-3 and AgRP:POMC ratio there was a significant effect of physiological state ($P=0.019$ and $P=0.001$ respectively) and light/dark phase ($P=0.002$ and $P=0.001$ respectively). For AgRP and POMC there were significant physiological state by light/dark phase interactions ($P=0.017$ and $P=0.035$ respectively); for these variables, values (bars) without the same label (a or b) differ significantly ($P<0.05$).

Figure 2 Hypothalamic expression of NPY (A), POMC (B) and SOCS-3 (C) as a function of serum leptin concentration. In each case the lines show the best-fitting regression model. For NPY, there was a significant state and light/dark phase interaction ($P=0.02$); when adjusted for this interaction, there was a significant correlation ($P=0.037$) between NPY and leptin. For both POMC and SOCS-3 there was a significant effect of state ($P<0.001$, $P=0.002$ respectively), but no effect of light/dark phase on the regressions. When adjusted for state effect, there was a significant correlation between the POMC and leptin ($P=0.007$), and between SOCS-3 and leptin ($P=0.004$).

changes in SOCS-3 reflect especially the status of the POMC neurones. Accordingly, multiple regression analysis of SOCS-3 as a function of POMC and NPY showed that 62% ($P<0.001$) of variation in SOCS-3 expression

could be explained by changes in POMC expression, with only an additional 5% ($P=0.039$) of variation being explained by changes in NPY expression. The decrease in SOCS-3 expression during the dark again suggests a

Figure 3 Hypothalamic expression of POMC as a function of SOCS-3. The four lines show the best-fitting regression model. There was a significant state and light/dark phase interaction ($P=0.017$); when adjusted for this interaction, there was a significant correlation ($P<0.001$) between the two variables.

constraint on leptin signalling through POMC neurones. Furthermore, the apparent failure of leptin to increase POMC expression at night is not due to increased expression of SOCS-3.

Effects of lactation on hypothalamic gene expression: relationship to leptin and food intake

Here, lactating rats showed increased hypothalamic NPY expression and decreased POMC during the day as compared with non-lactating rats. This agrees with previous findings (Ciofi *et al.* 1991, Pelletier & Tong 1992, Smith 1993, Malabu *et al.* 1994, Pape & Tramu 1996, Wilding *et al.* 1997, Li *et al.* 1998, Chen *et al.* 1999, Smith & Grove 2002, Garcia *et al.* 2003) and is consistent with roles for both peptides in the hyperphagia. We did not find increased AgRP expression (measured in the whole hypothalamus) in the light phase, perhaps because the previously reported increase in AgRP during the daytime in lactating rats was confined only to some regions of the arcuate nucleus (Chen *et al.* 1999). However, AgRP expression was increased in the dark in our lactating rats. The fact that POMC expression did not decrease at night in lactating rats may be due to the level already being reduced in the daytime. Thus lactation alters both the expression of these three neuropeptide genes and also changes the diurnal pattern of expression of AgRP and POMC.

The mechanisms regulating hypothalamic neuropeptide gene expression during lactation have not been resolved,

but both metabolic signals, such as leptin, and other factors, such as the suckling stimulus, are thought to be involved (Smith & Grove 2002, Vernon *et al.* 2002). The increased NPY expression could be at least partly due to the hypoleptinaemia. However, when changes during the dark as well as the light are taken into consideration, changes in AgRP and POMC expression cannot be directly related to changes in leptin, implying that other factors are probably more important in regulating expression of these two genes.

The effect of lactation on hypothalamic SOCS-3 expression has not been described previously. As SOCS-3 is an inhibitor of leptin signalling (Björbaek *et al.* 1999), an increase in expression could result in diminished leptin signalling and so facilitate hyperphagia. However, SOCS-3 expression fell during lactation, consistent with the hypoleptinaemia, but potentially enhancing the ability of neurones to transmit leptin signalling, and so is unlikely to contribute to the hyperphagia.

While we measured total food intake per day (14.3 ± 1.7 and 58.2 ± 3.8 g/rat for non-lactating and lactating rats respectively), we did not measure the proportion consumed during the light and dark periods in the present study. However, our own and other studies have shown that non-lactating and lactating rats consume respectively about 85 and 65% of their food at night (Strubbe & Gorissen 1980, Munday & Williamson 1983, Pickavance *et al.* 1996). From this, we estimate that our non-lactating rats would have eaten about 2 and 12 g/12 h during the light and dark periods respectively, and the lactating rats about 20 and 38 g/12 h during these periods. The expression of NPY, AgRP or POMC did not reflect this pattern of changes (Fig 1A–C). By contrast, changes in the AgRP:POMC ratio (Fig. 1E) showed a close parallel with this pattern of food intake. Levels of mRNA do not necessarily reflect the amount of these neuropeptides, but the strength of the relationship strongly suggests that the balance of agonist versus antagonist signalling (rather than changes in either alone) through the MC4 receptor could have a major role in controlling both the diurnal feeding rhythm and also hyperphagia in lactation. In addition, increased expression of NPY may contribute to the hyperphagia of lactation, but changes in NPY expression would not appear to contribute to night-time eating.

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