Neuropeptide Y gene expression in lines of mice subjected to long-term divergent selection on fat content

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ABSTRACT

Lines of mice have been developed in our laboratory by divergent long-term selection for body fat content. This has resulted in a fivefold (23% vs 4%) higher fat percentage in the Fat line at 14 weeks of age, with little difference between the Fat and Lean lines in fat-free body weight. As part of an approach to characterize the physiological mechanisms underlying these different phenotypes, neuropeptide Y (NPY) mRNA levels in the hypothalamus and cerebral cortex of ad libitum-fed and fasted mice of the Fat and Lean selected lines were measured. Significant differences in NPY gene expression were confined to the hypothalamus. Under ad libitum-fed conditions, hypothalamic NPY mRNA levels did not differ significantly between the Fat and Lean lines. After an overnight fast of 18–20 h, hypothalamic NPY mRNA levels were increased significantly \((P<0.05)\) by 31% in Lean animals relative to fed mice from the same line. However, fasting did not significantly stimulate NPY gene expression in the Fat line. Most plasma leptin measurements in the Lean line fell below the sensitivity threshold of the assay \((0.1 \text{ ng/ml})\), but levels in the Fat line were at least 30 to 50 times higher under fasted and fed conditions respectively. After fasting, plasma leptin levels in the Fat line decreased significantly \((P<0.05)\) by 48%. Thus, unlike the situation in other rodent models, obesity in the Fat line is not associated with increased hypothalamic NPY mRNA levels in the ad libitum-fed state. The decreased sensitivity of hypothalamic NPY gene expression to fasting in the Fat line is consistent with an inhibitory effect of higher circulating leptin levels.

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INTRODUCTION

Knowledge of the regulation of body weight in mammals has been advanced by the availability of several well-defined genetic models of obesity in rodents. For example, in mice there are at least six different single-gene mutations on five chromosomes that are associated with obesity syndromes. These include obese \((Lep, \text{ formerly } ob)\), diabetes \((Lepr, \text{ formerly } db)\), tubby \((tub)\), carboxypeptidase E \((Cpe, \text{ formerly } fat)\), adult obesity \((Ad)\), and the dominant yellow mutations at the agouti coat-colours locus \((A^Y, A^{VY})\) (Doolittle et al. 1996). Identification of the recessive gene responsible for obesity in the \(Lep^{ob}/Lep^{ob}\) (formerly \(ob/ob\)) mouse initiated a cascade of experiments that established leptin, the protein product of the \(Lep\) gene, as a hormonal feedback signal of body fat content to the brain (Zhang et al. 1994, Caro et al. 1996, Friedman & Halaas 1998). The obesity shown by \(Lep^{ob}/Lep^{ob}\) mice is due to a lack of circulating leptin as a result of the mutation. However, in other obese rodent models, circulating leptin levels are elevated in proportion to body fat stores, suggesting that obesity is associated with resistance to the weight-reducing effects of leptin (Caro et al. 1996). While it is known that leptin resistance in \(Lepr^{db}/Lepr^{db}\) (formerly \(db/db\)) obese mice and in the obese Zucker \(Lepr^{fa}/Lepr^{fa}\) (formerly \(fa/fa\)) rat arises from a defective leptin receptor (Chen et al. 1996, Chua et al. 1996, Lee et al. 1996, Da Silva et al. 1998), its basis in other rodent obesity models and in obese humans remains to be established.

Mouse lines have been selected in our laboratory for high and low body fat content from the same base population for approximately 60 generations.
At the age of selection of 14 weeks, the Fat line (F-line) has a fivefold higher percentage of body fat than the Lean line (L-line), and this ratio increases with age (Hastings et al. 1991, Bünger & Hill 1998). However, the lines differ little in fat-free body weight (Hastings & Hill 1989, Bünger et al. 1998a). In previous work with these selected lines, it has been shown that twice-daily intraperitoneal injections of leptin (5 mg/kg) for 15 days significantly reduced body weight, food intake and fatness in both F-line and unselected control animals (Bünger & Hill 1997). Although there were no significant differences in the response to leptin administration between the lines, the response of the F-line was much more variable, suggestive of inter-individual differences in leptin sensitivity. There was also a large difference in plasma leptin levels between the lines, with titres in the F-line being 60 to 300 times higher than those of the L-line (Bünger et al. 1998b).

To characterize further the physiological mechanisms underlying the differences in fat deposition between the F- and L-lines, our focus in the present study is on neuropeptide Y (NPY). NPY neurones in the hypothalamus represent one of several brain targets for leptin action. NPY is synthesized in the arcuate nucleus and released in and around the paraventricular nucleus where it stimulates feeding and promotes fat storage (Clark et al. 1984, White & Kershaw 1990, Kalra et al. 1991, Billington et al. 1994). NPY synthesis and release are inhibited by leptin, and the leptin receptor is co-expressed with NPY in arcuate nucleus neurones (Stephens et al. 1995, Mercer et al. 1996, Schwartz et al. 1996, Kotz et al. 1998, Schwartz et al. 1998). Consistent with a negative feedback regulation by leptin, NPY is over-expressed in rodent models of leptin deficiency (e.g. the Lepr<sup>ob</sup>/Lepr<sup>ob</sup> mouse) or defective leptin signalling (e.g. the Lepr<sup>dr</sup>/Lepr<sup>dr</sup> mouse) (Chua et al. 1991a, Wilding et al. 1993). Furthermore, in obese Lepr<sup>ob</sup>/Lepr<sup>ob</sup> rats, in which the leptin receptor is defective, NPY gene expression fails to increase following a fast as it does in lean controls (Sanacora et al. 1990). This suggests that the response of hypothalamic NPY gene expression to fasting may be an indicator of leptin sensitivity. In the present study, we compare NPY gene expression between our Fat and Lean selected mouse lines under ad libitum-fed and fasted conditions.

**MATERIALS AND METHODS**

**Selection lines**
The F- and L-lines were founded by divergent selection from a three-way cross of two inbred (CBA, JU) and one outbred (CFLP) line (Sharp et al. 1984). For the first 20 generations, selection was on the ratio of gonadal fat pad weight to body weight (BW) at 10 weeks of age in males with three replicate lines in each direction. Subsequently, the replicates were crossed and selection continued in a single replicate using the ratio of dry carcass weight to BW in males at 14 weeks as an indicator of fatness (Hastings & Hill 1989, Hastings et al. 1991). Animals from generation 65 of the selection lines were used in the present study. Selection was suspended from generations 53 to 59 while mouse stocks were transferred to a new mouse house.

**Animal husbandry**
Mice were fed ad libitum Rat and Mouse no. 3 diet (digestible crude (d. c.) oil: 3-9%; d. c. protein: 20-9%; starches: 27-3%; sugars: 11-2%; digestible energy 12-1 MJ/kg) from weaning onwards (Special Diet Services, Witham, Essex, UK) and maintained with controlled lighting (12 h light, 12 h darkness, lights on 0700 h) at a temperature of 21 ± 1 °C. The animals were kept after weaning in groups of 3 to 8 in plastic cages (MB1, Kents Plastics Ltd). The lids of all experimental cages were covered inside with metal plates to restrict lid-climbing activity and support fat aggregation.

**Experimental procedures**
The study was performed on male mice at 14–15 weeks of age from the F- (n=20) and L- (n=20) lines. Half the mice from each line were fasted for 17-20 h (1700 h-1300 h) and the other half had food available ad libitum. Water was freely available to all animals. Mice were killed by cervical dislocation between 1000 h and 1300 h, and the hypothalamus and a sample of cerebral cortex were rapidly dissected, frozen in liquid nitrogen, and stored at −70 °C. Plasma was collected from trunk blood and stored at −20 °C. Animal procedures were in accordance with United Kingdom Home Office regulations.

**Ribonuclease (RNase) protection assay**
NPY mRNA was quantified by RNase protection assay with a mouse NPY riboprobe. To produce the riboprobe, oligonucleotide primers based on the sequence for rat NPY mRNA (Higuchi et al. 1988) were designed to amplify a 231-base fragment extending from nucleotides 113 to 343. After PCR amplification, this fragment was inserted into the EcoRV site of pBluescript II SK+ (Stratagene, Cambridge, UK) and transformed into E. coli.
XL1-Blue MRF (Stratagene). The orientation and identity of positive clones were established by sequencing and restriction mapping. After linearization with EcoRI, a 308-base antisense mouse NPY riboprobe was transcribed from the T7 promoter of the plasmid using T7 RNA polymerase (Roche Diagnostics, Lewes, East Sussex, UK). The riboprobe was labelled with $^{32}$P-UTP (Amersham, Little Chalfont, Bucks, UK) to a specific activity of $8 \times 10^{17}$ Bq/mole. For use as an internal control, a 138-base, $^{32}$P-labelled riboprobe was transcribed from a pTRI-cyclophilin-mouse antisense control template (Ambion, Austin, TX, USA). Total RNA was isolated from the hypothalamus and cortex tissue samples using the reagent RNAzol B (TFL-Test Inc., Friendswood, TX, USA) according to the manufacturer’s instructions. The RNase protection assay was performed using the RPA II kit (Ambion) according to the manufacturer’s instructions. The RNase protection assay was performed using the RPA II kit (Ambion) according to the manufacturer’s instructions. The RNase protection assay was performed using the RPA II kit (Ambion) according to the manufacturer’s instructions. The RNase protection assay was performed using the RPA II kit (Ambion) according to the manufacturer’s instructions. Briefly, 15 µg total RNA and the labelled NPY and cyclophilin riboprobes were hybridized at 45°C overnight. Following digestion with RNases A and T1, samples were precipitated, resuspended in gel loading buffer, and loaded onto a 6% polyacrylamide gel containing 8 M urea. The gel was dried and exposed to a PhosphorImager plate (Molecular Dynamics, Sunnyvale, CA, USA) for 16 h. A representative assay is depicted in Fig. 1. Digitized images were captured with the PhosphorImager and densitometric quantification was performed by computer image analysis with the NIH Image program. Values for mean NPY mRNA levels were calculated from the ratio of NPY mRNA to cyclophilin mRNA in each individual sample. Owing to the limited number of samples that could be loaded onto the gel, the results were obtained from a total of eight separate assays. Following total RNA extraction from the hypothalamus tissue samples, two assays were performed to compare the F- and L-lines under fed and fasted conditions respectively. The RNA samples were then frozen at $-70^\circ$C until two further assays were performed three days later to compare fed and fasted conditions within each line. This procedure was then repeated for the cortex tissue samples.

**Carcass analysis**

Carcasses were freeze-dried to determine the individual dry weight (DW). Prediction of individual fat content was by regression on dry matter content (DW/BW) using an equation given by Hastings and Hill (1989) for fat content at 10 weeks: fat content (%) = DW/BW x 113–30/2. The fat-free body weight was estimated as body weight minus estimated fat content.

**Plasma leptin determination**

Plasma leptin levels were determined by a solid-phase sandwich enzyme immunoassay as described by Hotta et al. (1996).

**Statistical methods**

NPY data were analysed using the following model:

$$Y = M + G + L(G) + T + A + I(L) + e$$

where $M$ is an overall mean, $G$ is a genotype (line) effect (1–2), $L(G)$ is a litter within line effect, $T$ is the effect relating to the pre-treatment (fasted/fed), $A$ is an effect of the assay, $I$ is an individual effect (every individual was tested in two assays), and $e$ is the residual error. All effects except $L$, $I$ and $e$ were fitted as fixed, and the variance of $e$ was assumed to be constant in every assay. The analysis was undertaken with ASREML (Gilmour et al. 1997). Data were also log-transformed as there was a correlation between means and standard errors. However, because the untransformed and log-transformed data yielded the same results, we show only those derived from the untransformed data.

Data on body weights and composition, and on plasma leptin levels, were analysed with the same model but, instead of an assay effect, there was an effect of batch (B, in the freeze-dryer) for the dry matter and fat data and there was no individual effect. ANOVA was undertaken with GLM using

The sensitivity threshold of the leptin assay was 0·1 ng/ml. In total, 18 of 40 samples fell below this threshold. All were from the L-line, including all samples from the fasted group, and 8 of 10 mice from the fed group. Therefore the L-line provided data which were recorded as 0·1 ng/ml but can be expected to be lower than this threshold. Least square means for the leptin levels in the L-line were calculated using the above-described model, but statistical comparisons of the means were not undertaken.

RESULTS

Body weights and composition

Body weights and body composition of the animals used for the study are summarized in Table 1. The F-line mice had a fat content of 22% and the L-line a fat content of 2–3%, a difference similar to other fat records taken on these lines (Bünger & Hill 1998). In addition to being significantly fatter, the F-line was also significantly heavier than the L-line. However, the lines differed little in fat-free body weight, indicating that the body weight difference was due to fat content. The body weights of the fasted animals were 12 to 15% lower compared with the fed animals, probably reflecting the decreased content of the digestive tract.

NPY gene expression

Significant changes in NPY gene expression were restricted to the hypothalamus (Fig. 2). Thus, no significant effects of line or feeding/fasting were apparent in the cerebral cortex. Hypothalamic NPY mRNA levels were higher in the L-line, although significantly so only after fasting. Within each line, fasting significantly increased hypothalamic NPY mRNA by 31% in the L-line, but there was no significant difference between fed and fasted animals from the F-line.

![Figure 2](image-url)  
**Figure 2.** NPY mRNA levels in hypothalamus and cerebral cortex of Fat and Lean selected mice under *ad libitum*-fed conditions, and after a 17–20 h overnight fast. Total RNA was isolated from tissue samples and quantified by RNase protection assay with mouse NPY and cyclophilin riboprobes. Results (means ± s.e.m.) are expressed in arbitrary densitometric units and were calculated from the ratio of NPY mRNA to cyclophilin mRNA in each individual sample. Significant differences (*P*<0·05) from the lean fasted group for the hypothalamic samples are denoted by a. No significant differences were detected between groups for the cerebral cortex samples, as denoted by b.

### Table 1. Body weight, body composition, and plasma leptin levels of *ad libitum*-fed and fasted mice from the fat and lean lines

<table>
<thead>
<tr>
<th></th>
<th>Lean line</th>
<th>Fat line</th>
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<tbody>
<tr>
<td></td>
<td>Fed</td>
<td>Fasted</td>
</tr>
<tr>
<td></td>
<td>Mean*</td>
<td>s.e.m.</td>
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<tr>
<td></td>
<td></td>
<td></td>
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<tr>
<td>BW (g)</td>
<td>39·4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1·5</td>
</tr>
<tr>
<td>DM (g)</td>
<td>11·4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0·9</td>
</tr>
<tr>
<td>DM rel. (%)</td>
<td>28·9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0·7</td>
</tr>
<tr>
<td>Predicted fat (%)</td>
<td>2·3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0·8</td>
</tr>
<tr>
<td>Fat (g)</td>
<td>1·0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0·7</td>
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<tr>
<td>fBW (g)</td>
<td>38·4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0·9</td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>0·12</td>
<td>0·8</td>
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</table>

**a**Least square means and their standard errors. Means sharing a common superscript are not significantly different (*P*>0·05). Almost all plasma leptin values of the lean line were below the sensitivity threshold of the assay (0·1 ng/ml). Therefore they were not included in the statistical comparison of means. BW, body weight at 14–15 weeks of age: weight was taken before brain and blood were removed. fBW, fat-free body weight; DM, dry matter; DM rel, relative dry matter. Prediction of individual fat content was by regression on dry matter content (DW/BW) using an equation given by Hastings and Hill (1989) for fat content of fasted 10-week-old males. It was assumed that this regression also holds for 14–15 weeks of age. Overnight fasting does not influence predictions of fat percentage (L. Bünger, unpublished results). However, the body weights reflect the partial emptying of the digestive tract and, hence, the fat-free body weights.
Plasma leptin

Statistical analysis of leptin values was conducted on the data from the F-line only (Table 1), because almost all values in the L-line were at or below the sensitivity limit of the assay (0.1 ng/ml). However, the highest leptin levels in the F-line were at least 30 (fasted) to 50 (fed) times higher than those in the L-line. Within the F-line, plasma leptin levels decreased significantly by nearly 50% after fasting.

DISCUSSION

This study demonstrates that NPY gene expression in the hypothalamus is higher in the L-line than in the F-line, although only significantly so in fasted animals. Furthermore, while the L-line mice responded to fasting by significantly increasing hypothalamic NPY gene expression, no significant increase was observed in the F-line. In contrast to the hypothalamus, no significant differences in NPY mRNA levels as an effect of selected line or of feeding regimen were observed in the cerebral cortex. This confirms previous observations that changes in NPY gene expression associated with energy balance regulation occur specifically in the hypothalamus and not in other brain regions (e.g. White & Kershaw 1990, Sanacora et al. 1990, Chua et al. 1991b).

The similarity of hypothalamic NPY mRNA levels between the ad libitum-fed F and L mice indicates that the regulation of NPY gene expression in the F-line may differ from that in other well-studied rodent models of obesity. For example, when ad libitum-fed fat and lean lines of Lepr<sup>b</sup>/Lepr<sup>b</sup> rats, Lepr<sup>b</sup>/Lepr<sup>b</sup> mice, and Lepr<sup>a</sup>/Lepr<sup>a</sup> mice are compared, hypothalamic NPY mRNA is significantly higher in the obese animals (Sanacora et al. 1990, Chua et al. 1991a, Wilding et al. 1993, Stephens et al. 1995). In general, the overexpression of NPY in these rat and mouse models is believed to arise primarily from little or no negative leptin feedback signalling owing to mutation of the leptin gene (Lepr<sup>b</sup>/Lepr<sup>b</sup> mice) or the leptin receptor (Lepr<sup>a</sup>/Lepr<sup>a</sup> rats and Lepr<sup>b</sup>/Lepr<sup>b</sup> mice). Consistent with this, leptin administration reduces hypothalamic NPY mRNA in Lepr<sup>b</sup>/Lepr<sup>b</sup> but not Lepr<sup>b</sup>/Lepr<sup>b</sup> mice (Stephens et al. 1995, Schwartz et al. 1996). It is known from previous studies (Bünger & Hill 1997, Bürger et al. 1998b) that immunoreactive leptin is detectable in the plasma of the F-line, and that the majority of these animals are capable of responding to systemic leptin injections. The absence of increased NPY expression in the ad libitum-fed F mice in the present study provides further suggestive evidence that these animals do not have major lesions in the leptin or leptin receptor genes.

Obesity in the F-line differs from that in Lepr<sup>b</sup>/Lepr<sup>b</sup> mice and Lepr<sup>a</sup>/Lepr<sup>a</sup> rats in that it is not accompanied by hyperphagia (L Bünger, unpublished data). The fact that the F animals are not hyperphagic, and do not overexpress NPY mRNA in ad libitum-fed obese animals compared with lean controls, even when the obesity is associated with hyperphagia (e.g. Wilding et al. 1992, Mantzoros et al. 1998). Moreover, NPY gene knockout mice showed no differences from controls after challenges with fasting, chemical lesions, high fat diets, or genetic lesions (Hollopeter et al. 1998, Palmiter et al. 1998), and NPY deficiency only partly ameliorated the hyperphagia, obesity and endocrine abnormalities in the Lepr<sup>b</sup>/Lepr<sup>b</sup> mouse genetic background (Palmiter et al. 1998). Collectively, these results together with those from the present study suggest that an obese phenotype can develop independently of mechanisms involving NPY.

One possible limitation of the ribonuclease protection assay method used in the present study is that it quantified changes in NPY mRNA over the hypothalamus as a whole. It is, therefore, possible that differential NPY gene expression between individual hypothalamic nuclei may have been masked. For instance, in a recent <i>in situ</i> hybridization study of lethal yellow (A<sup>y</sup>) and melanocortin-4-receptor knockout mice, NPY was overexpressed in the dorsal medial hypothalamic nucleus of obese animals, but not in the arcuate nucleus (Kesterson et al. 1997). It cannot be ruled out that this was also the case in the present study.

The effect of fasting on the L-line was to increase hypothalamic NPY gene expression by 31% compared with the ad libitum-fed group. Increased hypothalamic NPY mRNA after fasting is a well-established phenomenon in non-obese rats and mice (White & Kershaw 1990, Chua et al. 1991b). In contrast, fasting failed to increase hypothalamic NPY mRNA in the F-line. There are two possible explanations for this observation. First, an insensitivity of hypothalamic NPY gene expression or peptide content to fasting is a consistent finding in obese rodents with mutations in the leptin receptor gene, such as Lepr<sup>a</sup>/Lepr<sup>a</sup> and JCR:LA corpulent rats and Lepr<sup>b</sup>/Lepr<sup>b</sup> mice (Sanacora et al. 1990, Williams et al. 1992, Chua et al. 1991a). In normal rodents, falling leptin levels appear to be at least partly responsible for the stimulation of NPY gene expression by fasting (Ahima et al. 1996). This
mechanism may be disrupted in genetically obese rodents that display leptin resistance because deficiencies in the leptin signalling system do not allow falling leptin levels to be detected. A blunted NPY response to fasting, as observed in the F-line in the present study, could therefore be interpreted as a marker of leptin resistance. However, the F-line mice did not differ significantly from the L-line in their response to systemic leptin injections (Bünger & Hill 1997) suggesting that they are not, in fact, markedly leptin-resistant. It therefore seems likely that the NPY neurones in the F-line mice have retained sensitivity to leptin, even though the animals are obese. Leptin titres in the F-line were at least 50 times higher than those in the L-line in the fed state and 30 times higher in the fasted state. It follows that a second, more likely, explanation for why NPY gene expression was less responsive to fasting in the F-line is that the higher circulating leptin levels in these mice inhibited NPY gene expression. This may have contributed to the trend for NPY mRNA levels to be lower in the F-line in the fed state. Moreover, while leptin levels did decrease after fasting in the F-line, they may still have been high enough to blunt an increase in NPY gene expression during fasting. A sensitivity of NPY neurones to high circulating leptin also appears to have been retained in obese UCP-DTA mice, even though they display peripheral leptin resistance with respect to body weight, food intake and glucose homeostasis (Mantzoros et al. 1998).

In summary, the lack of a significant difference in hypothalamic NPY mRNA levels between the F- and L-lines under ad libitum-fed conditions does not support a role for NPY as a primary determinant of obesity in these mice. The decreased sensitivity of NPY gene expression to an acute fast in the F-line is consistent with a negative feedback effect of higher circulating leptin levels.

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