A role for NPY overexpression in the dorsomedial hypothalamus in hyperphagia and obesity of OLETF rats

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Bi, Sheng, Ellen E. Ladenheim, Gary J. Schwartz, and Timothy H. Moran. A role for NPY overexpression in the dorsomedial hypothalamus in hyperphagia and obesity of OLETF rats. Am J Physiol Regulatory Integrative Comp Physiol 281: R254–R260, 2001.—Otsuka Long-Evans Tokushima Fatty (OLETF) rats lacking CCK-A receptors are hyperphagic, obese, and diabetic. We have previously demonstrated that these rats have a peripheral satiety deficit resulting in increased meal size. To examine the potential role of hypothalamic pathways in the hyperphagia and obesity of OLETF rats, we compared patterns of hypothalamic neuropeptide Y (NPY), proopiomelanocortin (POMC), and leptin receptor mRNA expression in ad libitum-fed Long-Evans Tokushima (LETO) and OLETF rats and food-restricted OLETF rats that were pair-fed to the intake of LETO controls. Pair feeding OLETF rats prevented their increased body weight and elevated levels of plasma insulin and leptin and normalized their elevated POMC and decreased NPY mRNA expression in the arcuate nucleus. In contrast, NPY expression was upregulated in the dorsomedial hypothalamus (DMH) in pair-fed OLETF rats. A similar DMH NPY overexpression was evident in 5-wk-old preobese OLETF rats. These findings suggest a role for DMH NPY upregulation in the etiology of OLETF hyperphagia and obesity.

cholecystokinin; cholecystokinin-A receptor; satiety; energy balance; in situ hybridization

The BRAIN-GUT PEPTIDE CCK is a peripheral satiety molecule that functions in the short-term control of food intake (12). CCK reduces meal size (21) and elicits the earlier appearance of a behavioral satiety sequence (3). The feeding-inhibitory effects of both exogenously administered and endogenously released CCK are mediated through their interaction with CCK-A receptors (27).

Otsuka Long-Evans Tokushima Fatty (OLETF) rats, an animal model of obesity and non-insulin-dependent diabetes mellitus (NIDDM) (19), have been shown to have a 6.8-kb deletion in the CCK-A receptor gene, resulting in the absence of CCK-A receptors (38). We have demonstrated that the hyperphagia in OLETF rats is characterized by a significant increase in meal size and have suggested that the lack of CCK-A recep-

tors in OLETF rats results in a peripheral satiety deficit leading to their hyperphagia and obesity (28).

Much of the recent work investigating controls of food intake and energy balance has focused on the hypothalamus. Leptin, a hormone produced in adipose tissue, acts as a feedback signal to the hypothalamus, playing a fundamental role in maintaining energy homeostasis (1, 11). Two primary target cellular populations within the hypothalamic arcuate nucleus (Arc) for leptin action in energy balance have been identified. These are neurons in the medial arcuate in which the orexigenic peptides neuropeptide Y (NPY) and melanocortin receptor antagonist agouti-related protein (AgRP) are colocalized (7, 16) and neurons in the lateral Arc in which the anorexigenic peptide precursor proopiomelanocortin (POMC) is expressed. Both of these neuronal populations express leptin receptor mRNA (8, 26). Leptin downregulates NPY/AgRP mRNA expression (1, 33, 36) and upregulates POMC mRNA expression (34, 40). Alterations in the activity of these signaling pathways have been shown to produce significant disruptions in energy balance (17, 36, 39, 45).

The current experiments were carried out to determine whether alterations in these hypothalamic signaling pathways may contribute to the hyperphagia and obesity in this CCK-A receptor spontaneous knock-out model. We have compared rates of weight gain, body fat development, plasma levels of leptin and insulin, and levels of hypothalamic NPY, POMC, and the long form of leptin receptor (Ob-Rb) mRNA expression in OLETF rats that had either ad libitum food access or that were pair-fed to amounts consumed by LETO control rats.

METHODS

Animals and pair feeding. Eighteen male OLETF and twelve age-matched male LETO rats were obtained as a generous gift of the Tokushima Research Institute, Otsuka Pharmaceutical, Tokushima, Japan. Rats were individually housed and maintained on a 12:12-h light-dark cycle (lights on at 7:00 AM) with tap water available ad libitum. In the first experiment, at 6 wk of age rats were placed in a pair-fed regimen. Six LETO and six normal-fed OLETF rats were maintained with ad libitum food access to standard chow. At 9:00

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in gradient ethanol, air-dried, and exposed with BMR-2 film (Kodak) for 1–3 days (4).

Quantitation. Quantitative analysis of the in situ hybridization was done with National Institutes of Health Scion Image software. Autoradiographic images were first scanned by Epson professional scanner (Epson) and saved in a computer for subsequent analyses with Scion image program using autoradiographic 14C microscales (Amersham) as a standard. Data for each animal were the mean density obtained from four to six sections reflecting the level of gene expression in a certain region. Data of OLETF rats were normalized to LETO controls as 100 in the Arc or 1 in the DMH.

For statistical analysis, data were analyzed using one-way ANOVA across the three experimental groups or Student’s t-test when only two groups were compared (P < 0.05 as a statistical difference).

RESULTS

Effects of pair feeding on body weight, body composition, and plasma leptin and insulin levels. Ad libitum-fed OLETF rats (OLETF-AF) gained ~26% more body weight than lean LETO controls during the 6-wk feeding experiments (Fig. 1 and Table 1). As shown in Table 1, at the end of the 6-wk period, OLETF-AF animals had 57% more epidydimal WAT (P < 0.05) and 41% more interscapular BAT than LETO controls (P < 0.05). OLETF-AF rats were also hyperleptinemic and hyperinsulinemic. Levels of both plasma leptin and insulin were significantly increased about 2.7- and 2.5-fold, respectively, in OLETF-AF rats compared with LETO controls (Table 1). When OLETF rats had their daily food intake limited to the amounts that were consumed by the LETO controls (OLETF-PF), their abnormal body weight, body composition, and plasma leptin and insulin levels were normalized. Body weight for the OLETF-PF rats did not differ from that of the LETO controls (Fig. 1 and Table 1). OLETF-PF controls had the same amounts of WAT and BAT as lean LETO controls, and the level of plasma insulin in OLETF-PF rats was similar to that in LETO controls. Plasma leptin was significantly reduced in the pair-fed rat compared with the LETO control (Table 1).

Table 1. Effects of pair feeding on body weight, body composition, plasma leptin, and insulin levels in OLETF rats

<table>
<thead>
<tr>
<th></th>
<th>LETO Ad Libitum Fed</th>
<th>OLETF Ad Libitum Fed</th>
<th>OLETF Pair-fed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>334 ± 6</td>
<td>345 ± 13*</td>
<td>324 ± 3</td>
</tr>
<tr>
<td>Left epidydimal WAT, g</td>
<td>2.35 ± 0.11</td>
<td>3.7 ± 0.23*</td>
<td>1.89 ± 0.13</td>
</tr>
<tr>
<td>Interscapular BAT, g</td>
<td>0.772 ± 0.047</td>
<td>1.09 ± 0.05*</td>
<td>0.88 ± 0.06</td>
</tr>
<tr>
<td>Plasma insulin, ng/ml</td>
<td>1.61 ± 0.24</td>
<td>3.99 ± 0.62*</td>
<td>1.54 ± 0.27</td>
</tr>
<tr>
<td>Plasma leptin, ng/ml</td>
<td>6.78 ± 0.72</td>
<td>18.5 ± 1.9*</td>
<td>3.2 ± 0.52*</td>
</tr>
</tbody>
</table>

Values are means ± SE. *Significant difference from LETO controls (P < 0.05). OLETF: Otsuka Long-Evans Tokushima Fatty; LETO, Long-Evans Tokushima; WAT and BAT, white and brown adipose tissue, respectively.

AM each day, six additional pair-fed OLETF rats were supplied an amount of chow equal to the prior day’s average daily chow intake of LETO rats maintained on ad libitum chow access. Body weight was measured daily. At the end of 6 wk, all rats were killed between 9:00 and 11:00 AM for evaluations of the amounts of total epidydimal white adipose tissue (WAT) and interscapular brown fat (BAT) and plasma levels of leptin and insulin as previously described (32). Brains were rapidly frozen for subsequent analyses of gene expression. In the second experiment, six 5-wk-old OLETF rats and six age-matched LETO controls were killed for in situ hybridization determinations of brain hypothalamic neuropeptide gene expressions.

Cryosections and riboprobes. Brains were removed immediately after decapitation, frozen with icy acetone, cut at 14 μm in a coronal section with a cryostat, mounted on superfrost/plus slides (Fisher Scientific), and fixed with 4% paraformaldehyde. Four to six sections per brain were anatomically matched among animals for each hybridization assay in the same condition.

The plasmids of POMC and NPY (generous gifts of Dr. R. Seeley and D. Baskins) and Ob-Rb (a generous gift of Drs. C. Bjorbaek and J. Flier) (10) were linearized by recommended restricted enzymes. Antisense riboprobes were labeled with [35S]UTP (Amersham Pharmacia Biotech) by using in vitro transcription systems with appropriate polymerases according to the manufacturer’s protocols (Promega) and purified by STE select-D G-50 columns (Eppendorf-5 Prime) to yield a specific activity of 5 × 108 cpm/μg.

In situ hybridization. For in situ hybridization, frozen tissue sections were allowed to warm to room temperature, treated with acetic anhydride and ethanol, and incubated in hybridization buffer containing 50% formamide, 0.3 M NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0, 1× Denhardt’s solution (Eppendorf), 10% dextran sulfate, 10 mM dithiothreitol, 500 μg/ml yeast tRNA, and 107 cpm/ml of [35S]UTP at 55°C overnight. After hybridization, the sections were washed three times with 2× standard sodium citrate (SSC), treated with 20 μg/ml RNase A (Sigma) at 37°C for 30 min, and then rinsed in 2× SSC twice at 55°C and washed twice in 0.1× SSC at 55°C for 15 min. Slides were dehydrated

Fig. 1. Effect of pair feeding on body weight in Otsuka Long-Evans Tokushima Fatty (OLETF) rats. Ad libitum-fed OLETF rats gained more weight than lean Long-Evans Tokushima (LETO) controls. Pair feeding reversed this excess body weight gain of OLETF rats to the same weight as LETO rats. Values are means ± SE, 6 animals in each group.
This may be a function of the partial food deprivation in the pair-fed rats. These data demonstrate that the obesity in CCK-A receptor-deficient OLETF rats and the alterations in plasma insulin and leptin are dependent on increased food intake.

Regulation of hypothalamic gene expression by pair feeding. Within the Arc, levels of both POMC and NPY gene expression differed significantly in ad libitum-fed OLETF and LETO rats as measured by in situ hybridization. POMC gene expression was 58% higher in OLETF rats than in LETO controls, and NPY mRNA was decreased by 38% in ad libitum-fed OLETF rats compared with LETO rats (Figs. 2 and 3). Restricting the food intake of OLETF rats to that of the LETO controls prevented the increased Arc POMC expression and restored the decreased Arc NPY mRNA levels (Figs. 2, A-F, and 3) such that Arc expression of NPY and POMC in pair-fed OLETF rats did not differ from those of the LETO controls. Transcription levels of hypothalamic Ob-Rb were not significantly altered in either ad libitum- or pair-fed OLETF rats relative to levels in LETO controls (Figs. 2, G-I, and 3). Taken together, these data suggest that the mediation of leptin signaling in the Arc is intact in OLETF rats and appropriately responding to their increased food intake and obesity. Thus OLETF rats do not appear to have a primary deficit involving Arc NPY or POMC signaling.

In contrast to the results in the Arc, the patterns of NPY mRNA expression in the dorsal medial hypothalamus (DMH) suggest a primary deficit in NPY signaling that may contribute to the hyperphagia and obesity in OLETF rats. NPY mRNA expression in the DMH was at the same low level in both groups of ad libitum-
fed rats, whereas NPY gene expression was significantly upregulated in pair-fed OLETF rats (Figs. 2, D-F, and 4). Despite similar levels of body weight, NPY mRNA expression within the DMH is eightfold higher in the food-restricted pair-fed OLETF rats relative to levels in the ad libitum-fed LETO controls. This overexpression is localized to the compact subregion of the DMH.

Overexpression of NPY in the DMH in young OLETF rats. To further address whether the increased DMH NPY was causally related to the hyperphagia and obesity in OLETF rats and not just secondary to the partial food deprivation resulting from pair feeding, we determined the levels of NPY gene expression in the DMH in OLETF and LETO rats at 5 wk of age, a time at which OLETF animals are just beginning to gain weight faster than LETO rats (weights at death 130 ± 0.6 g OLETF vs. 108 ± 1.1 g LETO, P < 0.05). In situ hybridization determinations demonstrated a significant 3.6-fold elevation in DMH NPY mRNA levels in young OLETF rats compared with the levels in lean LETO controls (Fig. 5, A, B, and D). Consistent with the small relative increase in body weight in OLETF rats, NPY gene expression in the Arc was slightly but significantly decreased at this age (Fig. 5, A-C). These data demonstrating increased DMH NPY expression preceding the development of the obesity in OLETF rats are consistent with a potential causative role for elevated NPY gene expression in the DMH in their hyperphagia and obesity.

DISCUSSION

The current data demonstrate that pair feeding OLETF rats to the amounts of food consumed by LETO controls normalizes body weight, body composition, and levels of plasma insulin and leptin. This finding is similar to the results of Okauchi et al. (31) who demonstrated the reductions in the obesity phenotype of OLETF rats in response to various levels of caloric restriction. These findings also confirm our previous suggestion that the obesity and NIDDM in OLETF rats with CCK-A receptor deficits are secondary to their hyperphagia (28).

The results from the in situ hybridization experiments demonstrated elevated POMC and reduced NPY mRNA expression in the hypothalamic Arc in free feeding, obese OLETF rats. Pair feeding OLETF rats to amounts consumed by LETO controls normalized Arc POMC and NPY gene expression as it normalized body weight, body fat, and plasma insulin and leptin levels. Activity in these neurons is likely responding to the elevated plasma leptin levels in the obese OLETF rats.
Leptin upregulates POMC expression and downregulates NPY expression in the Arc (1, 8, 33, 34). Thus Arc mRNA expression changes in the obese OLETF rats appear to reflect a response to their hyperphagia and increased body weight. These results for Arc NPY and POMC expression are similar to what has been demonstrated in response to involuntary overfeeding or overfeeding stimulated by chronic NPY administration (15, 25, 35). Leptin receptor expression was not altered in either the obese or pair-fed OLETF rats. These data suggest that the hyperphagia and obesity in OLETF rats are not due to primary deficits in a leptin signaling pathway involving Arc POMC or NPY.

Our finding of normally responsive Arc NPY and POMC expression in the ad libitum-fed OLETF rats is consistent with recent reports that document OLETF rat responses to exogenous leptin administration. Centralized administered leptin reduces food intake to the same degree in OLETF and LETO rats (30). The elevated levels of POMC expression in the obese ad libitum-fed OLETF rats may account for the reported reduction in $^{125}\text{I}$-labeled $[^{12}$Nle$^4$, d-Phe$^7$]-melanocyte stimulating hormone (MSH) melanocortin (MC) receptor binding in the ventromedial hypothalamus recently reported by Lindblom et al. (23). The upregulation of Arc POMC likely results in increased α-MSH production and release and a subsequent downregulation of MC receptor production.

Our data do suggest that OLETF rats may have a primary deficit in NPY gene expression in the DMH. DMH NPY is overexpressed in young OLETF rats prior to their developing obesity and is upregulated in adult OLETF rats in whom obesity has been prevented by pair feeding. A role for the DMH in food intake control has long been suggested by studies demonstrating that DMH-lesioned animals develop hypophagia and show reduced body weight (5). The DMH contains NPY cell bodies (2, 9), and an action of DMH NPY in food intake has been suggested by studies demonstrating increases in DMI NPY expression in lactation (37) and increases in DMH NPY concentration in response to intense exercise and food restriction (18, 22). There are several obese animal models in which elevated levels of DMH NPY mRNA expression have been noted. These include the lethal yellow Ay, MC 4 receptor knockout (20), tubby (14), diet-induced obese (13), and BAT-deficient obese mice (41). However, in these other obesity models, the increase in NPY gene expression in the DMH coincides with the development of, rather than preceding, the obesity as in the OLETF rat. Our current findings of the upregulation of NPY transcription in the DMH not only in pair-fed OLETF rats, but also in 5-wk-old OLETF animals, provide the first suggestion that increased DMH NPY expression may contribute to hyperphagia and obesity. Thus the elevated levels of NPY expression in the young OLETF rats drive their increased food intake. Only with hyperphagia and obesity are these DMH NPY expression levels then normalized.

The elevated levels of DMH NPY expression in the OLETF rats may be a direct consequence of the lack of CCK-A receptors. In intact animals, the DMH contains a dense population of CCK-A receptors (29, 44) and expresses high levels of CCK-A receptor mRNA (43). Additionally, the DMH is innervated by CCK-containing fibers (24, 42). A role for CCK acting in the DMH in food intake control has been suggested from studies of Blevins et al. (6), who demonstrated that CCK resulted in a greater inhibition of food intake when injected into the DMH than in any other brain area. In OLETF rats, NPY overexpression may result from the lack of an inhibitory signal mediated by CCK-A receptors within the DMH and contribute to their hyperphagia and obesity.

We have demonstrated previously that OLETF rats have a deficit in their ability to control the size of individual meals. In the absence of a functional peripheral CCK signaling pathway, meal size in OLETF rats is doubled. In response to this increase in meal size, meal number is decreased but not to a sufficient degree to prevent hyperphagia. We suggest that the inability of OLETF rats to compensate for their increased meal size may be secondary to the absence of DMH CCK-A receptors and the resulting increase in NPY expression. Thus the obesity in the OLETF rats may be the outcome of two regulatory disruptions, one depending on a peripheral within-meal satiety pathway and the other depending on a disruption in a central pathway critical to overall energy balance. These findings suggest that the CCK-A receptor knockout OLETF rat provides an important model for understanding interactions of the controls of individual meals and overall energy balance.

**Perspectives**

The Arc has been a focus of much of the recent work investigating hypothalamic controls of energy balance. The Arc is a major site for leptin actions. Leptin upregulates NPY AgRP-containing neurons and downregulates POMC-containing cells, and the actions of leptin on food intake have been proposed to depend on these actions. These leptin-sensitive cell bodies also have been postulated to play a role in the feeding response to food deprivation. Deprivation increases NPY and decreases POMC expression. In contrast to the well-identified actions of the NPY-containing cells within the Arc, the role of NPY cells in the DMH in energy balance is less understood. DMH NPY expression or NPY protein levels have been demonstrated to increase when female rats are lactating and in response to exercise, suggesting a role for this cellular population in modulating energy balance in response to increased energy needs (22, 37). A further separation of function is between Arc NPY and DMH NPY in that the NPY cells in the compact region do not seem to contain leptin receptors. Elmquist et al. (10) demonstrated that the DMH distribution of leptin receptors is concentrated to the caudal and dorsal subregions rather than in the compact region. Although NPY pathways may be differentially regulated, the present data suggest that alterations in DMH NPY signaling...
can contribute to feeding and body weight dysregulation, especially in the context of alterations within meal satiety signaling. Thus this combination suggests an alternative pathway to obesity independent of alterations in leptin signaling.

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REFERENCES


