Peptides that Regulate Food Intake
Acute food deprivation and chronic food restriction differentially affect hypothalamic NPY mRNA expression

Sheng Bi, Benjamin M. Robinson, and Timothy H. Moran
Department of Psychiatry and Behavioral Sciences, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Submitted 20 December 2002; accepted in final form 26 June 2003


Most of the recent work investigating actions of NPY in the control of food intake and energy balance has focused on the arcuate nucleus. Arcuate NPY gene expression is modulated in response to alterations in energy balance. Leptin, a hormone produced in adipocytes, acts as a feedback signal to the hypothalamus, playing a fundamental role in maintaining energy homeostasis (15, 31). Arcuate NPY serves as one of the downstream mediators of leptin’s actions. These NPY neurons coexpress leptin receptor mRNA (25), and NPY gene expression is downregulated by leptin administration (27). Arcuate NPY mRNA expression is modulated over the diurnal cycle, reaching the highest levels shortly before dark onset, the time of greatest food intake, and is increased in response to fasting, a time when circulating leptin levels are low (1, 2).

In contrast, the role of DMH NPY in energy balance is poorly understood. Potential functions of DMH NPY have been suggested from a variety of recent findings. DMH NPY mRNA expression is induced in lactating rats (30), and both chronic food restriction and fasting-induced increases in running wheel activity have been shown to increase DMH NPY protein concentrations (23). Moreover, alterations in DMH NPY mRNA expression have been reported in several obesity models, including the lethal yellow (Ay), melanocortin-4 receptor (MC-4R) knockout (21), tubby (18), high-fat diet-induced (17), and brown adipose tissue-deficient obese mice (35). We recently demonstrated that NPY gene expression is greatly increased in the compact subregion of the dorsomedial hypothalamus (DMH) in response to chronic food restriction, but not in response to acute food deprivation. Leptin receptor expression was not affected by either treatment. Double in situ hybridization histochemistry revealed that, in contrast to the situation in the arcuate nucleus, NPY and leptin receptor mRNA-expressing neurons were not colocalized in the DMH. Together, these data suggest that arcuate and DMH NPY gene expression are differentially regulated. DMH NPY-expressing neurons do not appear to be under the direct control of leptin signaling.

HYPOTHALAMIC PEPTIDE SIGNALING systems play an important role in the controls of food intake and body weight. Neuropeptide Y (NPY) is a potent hypothalamic orexigenic peptide (13, 22, 33). Centrally administered NPY causes robust increases in food intake and body weight and, with chronic administration, can eventually produce obesity (32, 38). Although NPY immunoreactivity is widely distributed throughout the hypothalamus, NPY is primarily localized in neuronal populations within the arcuate nucleus and dorsomedial hypothalamus (DMH) (3, 12, 16). Both arcuate and DMH NPY neurons project to the paraventricular nucleus (PVN) and roles for both of these NPY neuronal populations in the controls of food intake and energy balance have been proposed (19, 24).
In addition to well-known leptin actions in the arcuate nucleus, leptin receptor mRNA is also expressed in the DMH (14, 26, 27). However, a role for leptin in the control of DMH neuronal activity has yet to be demonstrated. Alterations in either NPY expression in the leptin-deficient obese (ob/ob) mouse (21) or NPY peptide levels in the obese (cp/cp) corpulent (Koletsky) rat (36) with a null mutation of the leptin receptor (20, 34) have been noted in the arcuate nucleus but were not found in the DMH.

Data such as these suggest the possibility that the regulation of arcuate and DMH NPY mRNA expression may differ. A number of the studies demonstrating alterations in DMH NPY activity have employed paradigms involving prolonged alterations in energy balance: lactation, chronic food restriction, or prolonged obesity (8, 17, 18, 21, 23, 30, 35). Thus we hypothesized that DMH NPY signaling may play an important role in the long-term control of food intake and in response to situations of increased energy demand, and, in contrast to arcuate NPY signaling, DMH NPY signaling may not be under the control of leptin. In the present experiments, we sought to compare whether arcuate and DMH NPY mRNA expression were differentially regulated in response to acute food deprivation, a challenge that has been previously demonstrated to engage leptin-dependent systems (1, 28), and prolonged food restriction, a challenge that may engage additional regulatory systems. In addition to analyzing hypothalamic NPY systems, we also compared patterns of hypothalamic gene expression for proopiomelanocortin (POMC), melanocortin receptor antagonist agonist-related protein (AgRP), and leptin receptor in response to acute food deprivation and chronic food restriction by in situ hybridization techniques. As well, we determined rates of body weight loss and plasma levels of glucose, leptin, and insulin in acute food-deprived and chronic food-restricted rats. Moreover, we examined the localization of NPY and leptin receptor mRNA expression in the DMH to determine whether or not these are contained within the same neuronal populations.

MATERIALS AND METHODS

Animals and feeding challenges. Eighteen male Sprague-Dawley rats (Charles River Laboratories) weighing 200–225 g were individually housed and maintained on a 12:12-h light-dark cycle (lights on at 6:00 AM) in a temperature-controlled colony room. At the beginning of the experiment, rats were divided into three groups. One group of six rats had access to standard chow ad libitum for 14 days and served as ad libitum-fed controls. Food intake was measured daily. The second group of six rats was treated as the chronic food restriction group, and their daily food intake was limited to 70% of the amount that was consumed by ad libitum-fed rats. Their food was supplied each day 2 h before lights off. The regimen of food restriction lasted for 14 days. The third group of six rats was the acute food-deprivation group. They were maintained for 12 days with ad libitum food access and were food deprived for the final 48 h before death. All rats had tap water available ad libitum, and their body weights were measured daily. At the end of the experiments, all rats were killed between 9:00 and 11:00 AM. The rats were decapitated under ether inhalation anesthesia. Trunk blood was taken for evaluation of plasma levels of glucose (Glucometer Elite, Bayer), leptin, and insulin (rat leptin and insulin RIA kits, Linco Research), as previously described (8). Brains were removed and rapidly frozen for subsequent analyses of gene expression.

Cryosections and riboprobes. Coronal sections (14 μm) through the PVN, arcuate nucleus, and DMH were taken via cryostat, mounted on superfrost/plus slides (Fisher Scientific), and fixed with 4% paraformaldehyde. Six sections per brain were anatomically matched among animals for each hybridization assay in the same condition.

The plasmids of NPY and POMC (generous gifts of Drs. R. Seeley and D. Baskins; Ref. 4), AgRP (DNA containing exon 4 and 3′-untranslated region, GenBank accession #U89486, a generous gift of Dr. G. Ronnett), and the long form of leptin receptors (ObRb; a generous gift of Drs. C. Bjorbaek and J. Flier; Ref. 14) were linearized by appropriate restriction enzymes. Antisense riboprobes were labeled with [35S]UTP (Amersham Pharmacia Biotech) by using in vitro transcription systems with T7 or T3 polymerases according to the manufacturer’s protocols (Promega) and purified by Quick Spin RNA columns (Roche Diagnostics) to yield a specific activity of 5 × 106 cpm/μg.

In situ hybridization. As previously described (8), sections were treated with acetic anhydride and incubated in hybridization buffer containing 50% formamide, 0.3 M NaCl, 10 mM Tris-Cl, pH 8.0, 1 mM EDTA, pH 8.0, 1× Denhardt’s solution (Eppendorf), 10% dextran sulfate, 10 mM DTT, 500 μg/ml yeast tRNA, and 106 cpm/ml of [35S]UTP at 55°C overnight. After hybridization, the sections were washed three times with 2× 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 twice in 0.1× SSC at 55°C for 15 min. Slides were dehydrated and exposed with BMR-2 film (Kodak) for 1–3 days.

In situ hybridization histochemistry. To assess the overlap of NPY and ObRb mRNA expression, we carried out individual in situ hybridization experiments on adjacent sections using either NPY or ObRb riboprobes as described above. After exposure to the film, images from the adjacent sections were scanned by Epson Professional Scanner (Epson), stored in a computer, and composed into a compound image with Adobe Photoshop software (Adobe Systems). The subnuclear localization for NPY and ObRb mRNA expression was further evaluated by staining the sections with cresyl violet.

Double fluorescence in situ hybridization. Nonradioactive riboprobes of NPY and ObRb were labeled with digoxigenin-11-UTP and biotin-16-UTP, respectively, by the in vitro transcription systems (Promega), and a mixture of double NPY and ObRb probes was applied simultaneously onto brain sections. The in situ hybridization procedure followed a standard protocol as described above (8). After hybridization and posthybridization washes, brain sections were processed for staining with two different fluorochromes. For detecting digoxigenin-labeled NPY signals, a fluorescent antibody enhancer set for DIG Detection kit (Roche Molecular Biochemicals) was conducted, and three kinds of antibodies (mouse IgG anti-DIG, anti-mouse-Ig-DIG and anti-DIG-rodhamine each in final concentration 1 ng/μl) were sequentially applied to brain sections in orders subsequently each step following 3× wash with washing buffer according to the manufacturer’s protocol. After staining NPY mRNA, biotin-labeled ObRb mRNA was detected by using the tyramide signal amplification and enzyme-labeled fluorescent (ELF) substrate method (9). Briefly, a tyramide signal amplification biotin system kit
were a mean of the product of hybridization area croscales (Amersham) as a standard. Data for each animal (leptin, and insulin. Caused great weight loss. 

RESULTS

Effects of acute food deprivation or chronic food restriction on body weight and plasma levels of glucose, leptin, and insulin. As shown in Fig. 1, chronic food restriction resulted in a reduced rate of body weight gain, and acute food deprivation produced body weight loss over the period of 2-day food deprivation. At death, as shown in Table 1, acutely food-deprived rats weighed 17.9% less than ad libitum-fed controls ($P < 0.05$), and this deprivation period resulted in a reduction in circulating leptin levels from 6.66 ± 1.34 to 1.31 ± 0.03 ng/ml ($P < 0.05$). Plasma glucose concentration was significantly decreased from 206 ± 12.1 to 86 ± 13.6 mg/dl ($P < 0.05$), and insulin levels fell from 2.53 ± 0.24 to 0.16 ± 0.06 ng/ml ($P < 0.05$). Chronic food restriction resulted in 27.3% less body weight gain than that of ad libitum-fed controls ($P < 0.05$), and at death these rats weighed 9.4% less than the acute food-deprivation group ($P < 0.05$). Chronic food restriction also significantly reduced plasma leptin levels from 6.66 ± 1.34 to 2.17 ± 0.33 ng/ml ($P < 0.05$), a level not different from that of the acutely food-deprived rats. However, in contrast to the reductions in response to food deprivation, rats with chronic food restriction had normal plasma glucose and insulin levels (Table 1).

Alterations in hypothalamic gene expression in response to feeding challenges. Within the arcuate nucleus, both acute food deprivation and chronic food restriction resulted in significant changes in NPY and POMC gene expression as measured by in situ hybridization (Fig. 2). Arcuate NPY mRNA was increased 251 and 156% of ad libitum-fed control levels in food-deprived and food-restricted rats, respectively ($P < 0.05$ in both cases). Planned $t$ comparison revealed that the increase in response to food deprivation was significantly greater than that found in response to the restriction paradigm ($P < 0.05$). Arcuate POMC gene expression was decreased by both treatments, reduced by 22.6% in response to acute food deprivation and 26.4% in response to chronic food restriction ($P < 0.05$). As shown in Fig. 2, arcuate AgRP gene expression was only significantly affected by acute food deprivation, increasing to 228% of levels of ad libitum-fed controls. This elevation was significantly different from both the ad libitum-fed and chronic food-restriction levels ($P < 0.05$). Chronic food restriction caused a slight increase in arcuate AgRP mRNA expression, but that effect was not statistically significant compared with ad libitum-fed controls ($P > 0.05$).

In contrast to these patterns of changes in the arcuate nucleus, NPY gene expression in the DMH was significantly affected by chronic food restriction but not

Table 1. Effects of acute food deprivation and chronic food restriction

<table>
<thead>
<tr>
<th></th>
<th>Ad Libitum-Fed</th>
<th>Food Deprived</th>
<th>Food Restricted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body wt, g</td>
<td>352 ± 7.6</td>
<td>289 ± 8.3*</td>
<td>256 ± 3.5†</td>
</tr>
<tr>
<td>Plasma glucose, mg/dl</td>
<td>296 ± 12.1</td>
<td>86 ± 13.6*</td>
<td>294 ± 9.6*</td>
</tr>
<tr>
<td>Plasma leptin, ng/ml</td>
<td>6.66 ± 1.34</td>
<td>1.31 ± 0.03*</td>
<td>2.17 ± 0.33*</td>
</tr>
<tr>
<td>Plasma insulin, ng/ml</td>
<td>2.53 ± 0.24</td>
<td>0.16 ± 0.06*</td>
<td>1.89 ± 0.24†</td>
</tr>
</tbody>
</table>

Values are mean ± SE. *Statistically significant difference from ad libitum-fed controls, and †statistically significant difference between food-restricted rats and food-deprived rats (1-way ANOVA and planned $t$ comparison, $P < 0.05$).

AJP-Regul Integr Comp Physiol • VOL 285 • NOVEMBER 2003 • www.ajpregu.org
by acute food deprivation. As shown in Fig. 3, chronic food restriction significantly increased DMH NPY gene expression relative to that of both the ad libitum-fed and acute food-deprivation groups ($P < 0.05$). Acute food deprivation did not significantly alter DMH NPY mRNA expression.

In contrast to these changes in NPY and AgRP mRNA expression, there were no significant differences in either arcuate or DMH ObRb gene expression among the three groups. Relative ObRb mRNA levels were $100 \pm 15.2$, $77.4 \pm 15.6$, and $94.7 \pm 17.8$ in the arcuate nucleus (1-way ANOVA, $P > 0.05$) and $100 \pm 11.5$, $80.1 \pm 4.9$, and $92.9 \pm 5.8$ in the DMH (1-way ANOVA, $P > 0.05$) in ad libitum-fed, food-deprived, and food-restricted rats, respectively.

**Histochemical relationships between NPY and ObRb in the DMH.** The differential effects of food deprivation and food restriction on NPY gene expression in the arcuate nucleus and the DMH suggest that these NPY neuronal populations may be differentially regulated. Both nuclei have been shown to express ObRb receptors (14, 26). Although ObRb has been shown to be expressed in NPY-containing neurons in the arcuate nucleus (25), whether DMH NPY containing neurons also express ObRb has not been established. As demonstrated in Fig. 4, in contrast to the coexpression of NPY and ObRb mRNAs in the arcuate nucleus, DMH NPY and ObRb mRNAs were not expressed in the same neurons within the DMH, and their expressions were found in distinct subregions. NPY gene expression was localized to the compact subregion of the DMH, whereas ObRb gene expression was found in more ventral subregions (Fig. 4).

**DISCUSSION**

The present data demonstrate that both acute food deprivation and chronic food restriction reduce body weight and circulating leptin levels and have differential effects on hypothalamic peptide gene expression. Both treatments elevate arcuate NPY and decrease arcuate POMC gene expression. Acute food deprivation resulted in increased arcuate AgRP mRNA levels. In contrast, chronic food restriction resulted in elevated DMH NPY gene expression. The alterations in arcuate gene expression are consistent with the alterations in circulating leptin levels. The chronic restriction-induced increase in DMH NPY gene expression is likely to be independent of leptin changes—NPY and ObRb mRNA were not coexpressed in a single neuronal population and their expressing neurons occurred in different DMH subregions. Furthermore, although circu-

![Fig. 2. mRNA levels of neuropeptide Y (NPY), proopiomelanocortin (POMC), and agouti-related peptide (AgRP) in the arcuate nucleus (ARC). In the ARC, NPY gene expression was increased in both food-deprived and -restricted rats, whereas POMC mRNA levels were reduced in response to both feeding challenges. Levels of ARC AgRP mRNA were increased in food-deprived but not in food-restricted rats. Relative mRNA levels were normalized to ad libitum-fed controls as 100%. Values are means ± SE, $n = 6$ per group. *$P < 0.05$ compared with ad libitum-fed controls; †$P < 0.05$ comparison of acute food deprivation vs. chronic food restriction (1-way ANOVA across the 3 groups and planned $t$ comparison).](http://ajpregu.physiology.org/)

![Fig. 3. Levels of NPY mRNA expression in the dorsomedial hypothalamus (DMH). Expression of NPY mRNA was detected in the DMH in rat brain by in situ hybridization determination (A) but not found in sections with RNase treatment (B). DMH NPY gene expression was upregulated in food-restricted rats (D and E), but not altered significantly in acute food-deprived rats (C and E) compared with ad libitum-fed controls (A and E). Relative mRNA levels were normalized to ad libitum-fed controls as 100%. Data are means ± SE, $n = 6$ per group. *$P < 0.05$ compared with ad libitum-fed controls; †$P < 0.05$ compared with the acute food-deprivation group (1-way ANOVA across the 3 groups and planned $t$ comparison). Bar = 550 μm.](http://ajpregu.physiology.org/)
lating leptin levels were decreased in response to both food deprivation and food restriction, ObRb gene expression was not significantly changed in either situation. Together, these data suggest multiple controls of hypothalamic peptide systems involved in feeding control.

Aspects of these data are consistent with previous findings demonstrating that treatments that decrease endogenous leptin levels result in alterations in patterns of hypothalamic gene expression that promote behavioral and metabolic responses to increase food intake and decrease energy expenditure (1, 28). Thus both acute food deprivation and chronic food restriction result in increases in arcuate NPY and decreases in arcuate POMC gene expression. Prior work has demonstrated that arcuate NPY and POMC containing neurons coexpress leptin receptors (11, 25), and the present results are consistent with leptin mediation of these expression changes within the arcuate nucleus. Both treatments result in decreased circulating leptin levels and appropriate alterations in arcuate gene expression. Arcuate AgRP gene expression was only significantly affected by acute food deprivation. Food deprivation produced a larger change in arcuate NPY as well. Thus the lack of a significant effect of chronic food restriction on arcuate AgRP gene expression could have been due to the extent of the prolonged deprivation (restriction period) or the relative severity of the dietary treatments.

We did not detect any changes in ObRb gene expression in response to either the acute food deprivation or the chronic food restriction. Although a number of investigators have reported deprivation-induced increases in hypothalamic ObRb mRNA expression (5), this has not been a universal finding. Bennett et al. (6) reported increased ObRb mRNA expression in the thalamus but no changes in the hypothalamus in response to fasting in rats, and Cai and Hyde (10) found a significant decrease in ObRb mRNA levels in the hypothalamus of normal mice in response to 48 h of food deprivation. These inconsistencies in fasting-induced changes in ObRb mRNA expression in the hypothalamus may imply that the regulation of ObRb mRNA expression is subregion specific. Thus the regulation of ObRb expression at different anterior/posterior levels of the arcuate nucleus may be under the control of different mechanisms. This possibility merits further investigation.

In contrast to the finding that both acute food deprivation and chronic food restriction elevated arcuate NPY gene expression, only chronic food restriction affected DMH NPY gene expression. Chronic food restriction resulted in a 56% increase in DMH NPY mRNA levels within the compact subregion, whereas acute deprivation did not produce a significant increase. The overall pattern of results suggests the possibility that the control of DMH NPY gene expression is not leptin dependent. Circulating leptin levels...
were equally reduced by both food deprivation and restriction, but only chronic restriction affected DMH NPY gene expression. Consistent with this interpretation, our histochemical data demonstrated that the distribution of DMH NPY-expressing neurons did not overlap with the distribution of ObRb-expressing neurons, and we did not detect DMH neurons that coexpressed NPY and ObRb mRNA. NPY mRNA was expressed in the compact subregion of the DMH, whereas ObRb mRNA was expressed in the ventral DMH. This finding is consistent with the report by Elmquist et al. (14) that ObRb expression within the DMH is localized to caudal regions of the nucleus, ventral to the compact zone.

Our finding that DMH NPY expression is localized to the compact subregion is consistent with our previous data demonstrating elevated NPY gene expression in the compact subregion in OLETF rats lacking CCK-A receptors (8). Alterations in DMH NPY expression in response to a variety of treatments have been identified. Some of these changes have been localized to the compact subregion, whereas others appear to occur outside this area. In lactating rats, Smith (30) reported that, although there was a very slight increase in NPY mRNA expression in the compact area, DMH NPY mRNA expression was mainly induced in the diffuse portion of the DMH. During development, changes in DMH NPY mRNA expression have been localized to both the compact and noncompact subregions (29). Moreover, whereas alterations in DMH NPY expression have been reported in agouti and MC-4R knockout mice (21), as well as other obese models (17, 18), the exact localization of this expression was not noted in these reports. It is not yet clear whether NPY mRNA expression in different subregions of the DMH is differentially regulated.

A role for the DMH in food intake control was first suggested from the results of studies demonstrating that DMH lesions resulted in hypophagia and reduced body weight (7). More recently, DMH NPY gene expression or protein levels have been shown to vary as a function of lactational status (30) and in response to food restriction and fasting-induced exercise (23). As well, DMH NPY mRNA expression is significantly elevated in several obesity models (8, 17, 18, 21, 35). Thus our current data demonstrating that DMH NPY mRNA levels are affected by chronic food restriction but not by acute food deprivation are consistent with a role for DMH NPY gene expression in the long-term control of food intake and in response to situations of increased energy demand. Guan et al. (17) also reported increased NPY gene expression in the DMH in a mouse model of high-fat diet-induced obesity. How this finding relates to the regulation of DMH NPY expression in states of increased energy demands is not clear. The specific subregions of the DMH were not identified in the Guan et al. (17) study, raising the possibility that this increase in NPY expression occurred in a different area of the DMH than those that respond to increased energy demand.

In summary, we demonstrated that DMH NPY gene expression was increased in response to chronic food restriction but not acute food deprivation. These data suggest that NPY gene expression is differentially regulated in the arcuate nucleus and the DMH. Unlike arcuate NPY, DMH NPY may play an important role in maintaining energy homeostasis only in response to long-term alterations in energy intake or expenditure. Neuroanatomically, the distribution of DMH NPY-expressing neurons did not overlap with the pattern of ObRb gene expression, suggesting that DMH NPY is unlikely to be directly controlled by alterations in leptin signaling.

DISCLOSURES

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-19302 and DK-57609.

REFERENCES


24. Li C, Chen P, and Smith MS. Neuropeptide Y (NPY) neurons in the arcuate nucleus (ARH) and dorsomedial nucleus (DMH), areas activated during lactation, project to the paraventricular nucleus of the hypothalamus (PVH). Regul Pept 75–76: 93–100, 1998.