Neuropeptide Y in hypothalamic paraventricular nucleus: a center coordinating energy metabolism

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Departments of Research and Medicine, Minneapolis Department of Veterans Affairs Medical Center, Minneapolis 55417; and Departments of Medicine, Food Science and Nutrition, and Psychiatry, University of Minnesota, Minneapolis, Minnesota 55454

Billington, C. J., J. E. Briggs, S. Harker, M. Grace, and A. S. Levine. Neuropeptide Y in hypothalamic paraventricular nucleus: a center coordinating energy metabolism. Am. J. Physiol. 266 (Regulatory Integrative Comp. Physiol. 35): R1765–R1770, 1994.—Intracerebroventricular injection of neuropeptide Y (NPY) has two effects on energy metabolism in addition to increased feeding: decreased brown fat thermogenesis and increased white fat lipoprotein lipase (LPL) enzymatic activity. We hypothesized that the paraventricular nucleus (PVN) of the hypothalamus is the controlling neural site for these responses. We further hypothesized that NPY stimulation at PVN would reduce gene expression for the critical brown fat thermogenic protein, uncoupling protein (UCP), and increase gene expression for the key white fat storage enzyme, LPL. In the first experiment, three groups of rats received injections every 6 h for 24 h (6 injections total) into the PVN: 1) NPY (1 μg/1 μl injection) and ad libitum food; 2) NPY (1 μg/1 μl injection) and food restricted to control intake; 3) saline injection (1 μl) and ad libitum food. Both NPY-treated groups showed significant reductions (P < 0.05) in brown fat UCP mRNA levels and marked stimulation of LPL mRNA levels relative to controls. In the second experiment, four groups of seven rats had NPY injected into the PVN: 0 (vehicle control); 0.1 μg; 0.5 μg; and 1 μg. Injections were made every 6 h for 24 h. There was a dose-related reduction in UCP mRNA produced by the NPY treatment. NPY treatment increased LPL mRNA, but a smooth dosing effect was not evident. The observation that NPY in the PVN can coordinate more than one component of energy metabolism is significant when considered with many reports of responsiveness of NPY activity in the arcuate nucleus-PVN neural circuit to perturbations of energy balance such as fasting and feeding, diabetes, and genetic obesity. Taken together, the observations suggest the presence of a highly organized and comprehensive control element for energy metabolism that reaches far beyond simple appetite control.

thermogenesis; energy expenditure; neuropeptides; appetite; food intake

THERE IS LITTLE DOUBT that the central nervous system is an important regulator of feeding. There is also strong evidence that portions of energy metabolism, including most obviously energy expended by physical activity, can be regulated by the central nervous system (6). The central regulatory mechanisms for these two sides of energy balance are little understood but have traditionally been thought of as separate functions. Debate about whether perturbations in energy balance, such as obesity, are consequent to a deficit in appetite or a deficit in energy expenditure is commonplace. Yet there is evidence, from hypothalamic lesioning studies and from direct stimulation of several brain sites, that energy intake and energy expenditure may be coregulated (5, 7, 32). For example, ventromedial hypothalamic lesions produce dramatic increases in food intake but also reduce energy expenditure (30).

In considering brain regulation of energy metabolism, much of the focus has been on the rat hypothalamus. Recently, the possible role of peptide neurotransmitters in hypothalamic regulation has been explored. Considerable evidence now supports the role of neuropeptide Y (NPY) in stimulating feeding within the hypothalamus (10, 20, 27). Administration of NPY into the cerebroventricular system robustly stimulates feeding (20) and is the most potent known neural stimulus for feeding. Localization studies have indicated that the NPY effect on feeding occurs most prominently in the paraventricular nucleus (PVN) of the hypothalamus (26) or in the nearby perifornical area (28). Large concentrations of NPY are normally found at the PVN (9), and it is known that the PVN receives NPY bearing axonal projections from the arcuate nucleus (2, 9) and other brain sites. The highest concentrations of NPY mRNA expression are also found in the arcuate nucleus of hypothalamus (21). Recent evidence indicates that NPY concentrations in the PVN (24), NPY release at the PVN (16), and NPY mRNA levels in the arcuate nucleus (4) increase in response to food deprivation and subsequently normalize when food is provided.

We have previously found evidence that intracerebroventricular injection of NPY had two effects in addition to increased feeding (3): NPY independently decreased brown fat thermogenesis (uncoupling protein (UCP) functional activity as assessed by GDP binding) and increased white fat lipoprotein lipase (LPL) enzymatic activity. In unpublished data, we had also found that NPY given intracerebroventricularly reduced brown fat gene expression for the UCP and increased white fat gene expression for LPL. We hypothesized that the PVN of the hypothalamus is the controlling neural site for these responses. We further hypothesized that NPY stimulation would reduce gene expression for the critical brown fat thermogenic protein, UCP, and increase gene expression for the key white fat storage enzyme, LPL.

METHODS

Male Sprague-Dawley rats weighing 225 to 250 g were used in all experiments. Standard lighting and temperature conditions in a well-ventilated vivarium (12-h day, artificial light, lights on 0700–1900) were used and, except where otherwise indicated, the rats had free access to diet (Purina Defined Rat Chow) and water. The rats were individually housed in wire mesh cages. The experimental program was approved by the institutional Animal Studies Committee. All surgical proce-
duree and preparations, including central nervous system cannulations, were performed under sterile conditions using pentobarbital (Nembutal) anaesthesia. Because of potential interference by anesthetics in metabolic measurements, anesthetic use was not possible during killing. Rather, humane use of rapid guillotine was employed under the supervision of the institutional Animal Studies Committee.

Brain cannulas were constructed of 23-gauge stainless steel tubing (Small Parts). A stereotaxic instrument (David Kopf) was used to locate the PVN of the hypothalamus in three planes with respect to skull landmarks (bregma, ear-bar zero) and cannulate that structure. Coordinates are from the rat brain atlas of Paxinos and Watson (22) for paraventricular hypothalamic nucleus: 1.9 posterior; 0.5 lateral; 7.3 down. The cannula was held in place with stainless steel screws and dental acrylic cement applied to anchor the cannula to the screws. Cannulas were closed with a stainless steel styllet when not in use. The rat was allowed at least 7 days to recover from the cannulation procedure.

Injection cannulas are fabricated from 30-gauge stainless steel tubes which are cut so that when inserted to their maximum depth they protrude 0.5 mm beyond the tips of the guide cannulas. Injection cannulas are attached via polyethylene tubing to microsyringes (Hamilton, 10 μl). Injection volume was 1.0 μl, given slowly over 30 s. Cannulas are left in place an additional 15 s to allow diffusion from the tip. After injection, the cannula was withdrawn, the styllet was replaced, and the rat returned to its home cage.

After killing, the placement of cannulas was verified by histology. After at least 7 days Formalin fixation, the brains were frozen and 40-μm sections were cut corresponding to the brain atlas of Paxinos and Watson (22). These sections were stained and examined to verify cannula placement. Data from rats with incorrect placement were discarded.

In the first experiment, three groups of rats received injections every 6 h for 24 h (5 injections total) into the PVN: 1) NPY (1 μg/1 μl injection) and ad libitum food; 2) NPY (1 μg/1 μl injection) and food restricted to control intake; 3) saline injection (1 μl) and ad libitum food. The pair feeding was designed so that the rate of food consumption was monitored at intervals and provision of food to the yoked animals closely simulated that seen in saline controls. Rats were killed 2 h after the final injection and therefore 28 h after the first injection. Brown fat and white fat as well as the brain were collected for analysis. Brown fat mRNA was extracted and probed for UCP and β-actin expression. White fat mRNA was extracted and probed for LPL gene expression and β-actin expression. In this experiment, serum was also collected for measurement of glucose, insulin, and corticosterone. There were originally 24 rats with cannulas targeted for the PVN. After deleting animals because of health problems or incorrect cannula placement, we finished with 16 animals in the analysis.

In the second experiment, four groups of seven rats were injected with three different NPY doses (all dissolved in 1 μl vehicle) into the PVN. 0 (vehicle control), 0.1, 0.5, and 1 μg. Injections were made every 6 h for 24 h (total of 5 injections and 28 h). In this experiment food intake was uncontrolled. Rats were killed 2 h after the final injection. Brown fat, white fat, and brain were collected for analysis. Brown fat mRNA was extracted and probed for UCP. White fat mRNA was extracted and probed for LPL gene expression.

Glucose was measured by glucose oxidase method (Sigma, St. Louis, MO). Serum insulin was measured by radioimmunoassay (ICN Biochemicals, Costa Mesa, CA). Serum corticosterone was also measured by radioimmunoassay (ICN).

Total brown fat RNA was extracted by the rapid guanidine thiocyanate/phenolchloroform method (8). The total RNA was probed in a slot blot with cDNA clones for relevant proteins. Aliquots of brown fat mRNA were probed with a cDNA for UCP (UCP-365; generously provided by Dr. Daniel Ricquier (see Ref. 23)). White fat total RNA was similarly extracted by the rapid method and slot blotted. Aliquots of the white fat RNA were probed with a cDNA for UCP (generously provided by Dr. Robert Eckel of the University of Colorado). β Actin probe was commercially obtained (Oncor, Gaithersburg, MD). Probes were labeled by the random primer method (BioRad, Richmond, CA). All RNA from a single experiment was slot blotted onto a single filter, hybridized in a single vessel, and autoradiographed in a single cassette to assure comparability of group treatment. Three different quantities of each RNA sample were loaded onto each blot: 1, 2, and 4 μg. Control for RNA loading onto the slot-blots was provided by the ultraviolet shadowing technique, in which total unhybridized RNA is imaged (29). The different quantities of RNA loading provided a method of determining that autoradiographic exposure was in the linear range of the film, and also provided replicate data for each animal. The reported data generally use information from the 4-μg slots. Hybridization was quantitated in arbitrary optical density units by scanning densitometry (BioRad).

Data analysis was by analysis of variance (ANOVA) with post hoc testing by Scheffe’s procedure. All data are displayed as means ± SE.

RESULTS

Food intake (Fig. 1) was similar over the 26 h of study in controls and NPY-yoked groups (14.0 ± 4.4 and 10.2 ± 0.8 g/day, NS), while the NPY ad libitum group consumed significantly more (24.0 ± 2.3 g/day; P < 0.05 compared with control and yoked groups). The NPY feeding effect was manifest, and the success of the pair feeding is illustrated not only for the 24-h aggregate intake, but for intervening time points as well. The NPY ad libitum group was different by ANOVA from the other two groups at each time point: at 6 h F(2,13) = 64.206, P = 0.0001; at 12 h F(2,13) = 12.595, P = 0.0009; at 18 h F(2,13) = 0.072, P = 0.0137; and at 26 h F(2,13) = 6.583, P = 0.0106.

Body weights were measured before and after the treatment, and tissue weights of brown fat and heart.
Table 1. NPY into PVN: groups, body weights, and selected tissue weights

<table>
<thead>
<tr>
<th>Group</th>
<th>Pretreatment Body Wt, g</th>
<th>Posttreatment Body wt, g</th>
<th>Brown Fat Wt, mg</th>
<th>Heart Wt, mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5 257 ± 4</td>
<td>289 ± 12</td>
<td>207 ± 28</td>
<td>774 ± 23</td>
</tr>
<tr>
<td>NPY ad libitum</td>
<td>6 260 ± 5</td>
<td>280 ± 11</td>
<td>245 ± 28</td>
<td>772 ± 41</td>
</tr>
<tr>
<td>NPY restrict</td>
<td>5 253 ± 7</td>
<td>286 ± 8</td>
<td>162 ± 21</td>
<td>734 ± 52</td>
</tr>
<tr>
<td>ANOVA F Ratio</td>
<td>0.365</td>
<td>0.214</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P value</td>
<td>0.70</td>
<td>0.81</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of animals. There are no significant differences by ANOVA.

were made to ensure comparability of the groups (Table 1).

Brown fat total RNA samples from each of the treatment groups (n = 5 for saline, n = 6 for NPY ad libitum, n = 5 for NPY yoked) were hybridized in a slot blot to a radiolabeled cDNA probe for the UCP [UCP-365, Ricquier et al. (23)]. The blots were quantitated by videodensitometry and the data analyzed by ANOVA. Both NPY-treated groups showed significant reductions (P < 0.05) in uncoupling protein mRNA levels relative to controls (Fig. 2). Level of β-actin gene expression in the brown fat was similarly affected by the NPY/PVN treatments (control 5.97, NPY ad libitum 0.9, and NPY restricted 1.53 OD units; F = 41.452, P < 0.0001), indicating that the effect was tissue specific for brown fat rather than gene specific for UCP. NPY effects on gene expression for both proteins were so dramatic that a UCP/actin expression ratio was not interpretable.

Perirenal white fat was also obtained from each animal and total RNA extracted in the same way as for the brown fat. Aliquots of the white fat RNA were hybridized to a cDNA probe coding for LPL. The results indicate a marked stimulation of LPL mRNA levels by NPY administration into the PVN (Fig. 3A). White fat RNA was also probed with β-actin and here no effect was found (control 14.19, NPY ad libitum 13.08, and NPY restricted 17.43; F = 1.037, P = 0.3933), apparently indicating a gene-specific effect.

The specificity of the NPY/PVN effect on peripheral fat storage was further assessed by comparing the above-described stimulation of LPL gene expression in white fat to gene expression for LPL in another tissue where LPL plays a prominent role, skeletal muscle. Supraspinatus muscle was the tissue selected, and the results of slot blot hybridization with the cDNA for LPL are indicated in Fig. 3B. No influence of NPY administration into the PVN was found on this measure.

The physiological mediators linking NPY/PVN stimulation to alterations in peripheral energy metabolism are not well defined. In this study, we measured serum glucose, insulin, and corticosterone to consider a possible mediating role for these parameters.

The results (Table 2) are not consistent with an important role for these variables in explaining the

![Fig. 2. Effect of NPY administration into paraventricular nucleus (PVN) on brown fat uncoupling protein mRNA. Significant treatment effect by ANOVA: F(2,13) = 24.339, P = 0.0001. Both NPY groups are different from control by Fisher’s least-significant difference (LSD) and by Scheffe’s tests. The NPY groups are not different from one another.](http://ajpregu.physiology.org/)

![Fig. 3. Effect of NPY administration into the PVN on lipoprotein lipase (LPL) mRNA in white fat (A) and in supraspinatus skeletal muscle (B). In white fat there is a significant treatment effect by ANOVA: F(2,9) = 11.095, P = 0.0037. NPY groups are different from control but not from each other by Fisher’s LSD and Scheffe’s tests. In skeletal muscle, there is no effect by ANOVA.](http://ajpregu.physiology.org/)
Table 2. Serum values after NPY administration into PVN

<table>
<thead>
<tr>
<th>Glucose, mg/dl</th>
<th>Insulin, μU/ml</th>
<th>Corticosterone, ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>136±7</td>
<td>64.5±5.9</td>
</tr>
<tr>
<td>NPY ad libitum</td>
<td>132±3</td>
<td>131.2±9.8</td>
</tr>
<tr>
<td>NPY restrict</td>
<td>138±5</td>
<td>72.4±9.4</td>
</tr>
</tbody>
</table>

ANOVA F value

<table>
<thead>
<tr>
<th>Glucose</th>
<th>Insulin</th>
<th>Corticosterone</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.164</td>
<td>16.619</td>
<td>0.284</td>
</tr>
<tr>
<td>0.85</td>
<td>0.0003</td>
<td>0.758</td>
</tr>
</tbody>
</table>

Values are means ± SE.

observed effects on white and brown fat. Insulin was elevated relative to controls in the NPY ad libitum-fed group but not in the NPY-yoked group, while the NPY/PVN effect appeared in both NPY groups regardless of feeding status. We could detect no significant effect of NPY/PVN on corticosterone, though there may have been a trend toward higher values in the NPY-treated groups.

In experiment 2, we used a dose response to assess the significance of the NPY/PVN control system with respect to peripheral metabolic regulation. Doses lower than those in experiment 1 were given in the same number and for the same duration. For this experiment, no food intake-matched control was included. The result of treatment on feeding is shown in Fig. 4. NPY in doses of 0.5 and 1 μg, but not in a dose of 0.1 μg, given into PVN increased feeding over 24 h. The effect of NPY every 6 h for 24 h on UCP gene expression in brown fat is shown in Fig. 5. There is a dose-related reduction in UCP mRNA produced by the NPY treatment.

The effect of NPY into the PVN on white fat LPL gene expression is shown in Fig. 6. In this case, the NPY treatment clearly increased LPL mRNA, but a smooth dosing effect is less evident.

DISCUSSION

NPY administration into the PVN stimulated robust food intake (Figs. 1 and 4), in accord with previous studies suggesting that the PVN is a particularly sensitive site in mediating NPY-stimulated food intake (20, 26). Other data now support the role of the PVN in regulating food intake. Rats deprived of food are found to have increased NPY peptide levels in the PVN (24), as are animals in other negative energy balance states associated with increased feeding, such as diabetes (34). Restoration of food or amelioration of the negative energy balance state leads to reduction in PVN NPY levels (24, 34). NPY release by PVN in rats has been shown to rise before a scheduled meal and decline after onset of eating (16). NPY levels in PVN have been shown to peak before the onset of the natural feeding cycle in rats (15). We therefore believe that the PVN is a critical though perhaps not the sole site for mediating the effects of NPY on energy metabolism.

NPY stimulation of PVN reduced UCP gene expression to ~38% of control value. These data are consistent with our previous study (3) in which NPY given into the cerebral ventricles in a similarly designed food intake-matched experiment showed ~30% reduction in UCP protein activity via measurement of GDP binding to isolated brown fat mitochondria. In the first experiment, the NPY effect on UCP gene expression was independent of the parallel induction of feeding. Equivalent reductions in UCP gene expression were produced in the NPY PVN-stimulated groups with or without excess food intake (Fig. 2). The important effect on UCP gene expression, combined with previous data on UCP
function, makes it likely that the NPY/PVN treatment is producing similarly important reductions in brown fat thermogenesis, thus seriously limiting energy expenditure through this tissue. In rats, brown fat is an important site of nonshivering thermogenesis (14). Reduction of brown fat energy expenditure is therefore consistent with enhanced effectiveness of weight gain relative to food intake (27).

The second study (Fig. 5) indicates that the NPY/PVN stimulation has dose-dependent effects on UCP gene expression in brown fat. Such dose-response studies provide greater assurance that observed effects are physiological, rather than purely pharmacological phenomenon. How these doses of NPY compare to levels present normally in the relevant synapses remains conjectural, lacking a convincing technology to measure endogenous levels.

While NPY in the PVN increased food energy intake and decreased energy expenditure in brown fat, energy storage in white fat appears to be increased, as indicated by the increases in LPL gene expression in white fat. In the first experiment, NPY into PVN approximately doubled LPL gene expression in white fat (Fig. 3). The pair-fed group results indicate that augmented feeding produced by NPY is not necessary for the NPY-induced white LPL gene expression effect. These LPL gene expression results agree with previous studies in which NPY given intracerebroventricularly increased by two- to fourfold white fat LPL enzymatic activity (3). The results from the second experiment do not indicate a dose effect of NPY in the PVN for white fat LPL gene expression (Fig. 6). This disparity may reflect a difference in the function of the NPY/PVN control pathway of white fat activity relative to brown fat and feeding or may reflect difficulties in working with and extracting RNA from white fat. The tissue specificity of the NPY/PVN effect on LPL gene expression is indicated by the results of the LPL gene expression in skeletal muscle, where no difference was found. In muscle, LPL activity correlates generally with lipid oxidation and energy expenditure, in contrast to the fat storage expected in association with LPL activity in white fat. Because of the tissue specificity, we do not believe that the NPY/PVN effects on white fat reflect some nonspecific metabolic, neural, or assay process.

The data from these experiments are consistent with the hypothesis that the single stimulus of NPY at a single site, the PVN, can produce important effects on energy balance by complementary alterations in at least three independent components of energy metabolism: food intake, brown fat thermogenesis, and white fat storage activity. The other major finding of this investigation is that central nervous system stimulation by NPY in the PVN produced alterations in relevant peripheral gene expression. Linkage of NPY/PVN-induced feeding to NPY/PVN modulation of peripheral energy metabolism fits with an accumulating body of evidence that feeding behavior and peripheral energy metabolism are regulated in complementary fashion by control elements in the central nervous system, particularly in the hypothalamus (5). This perspective is consistent with reports (18) of NPY affecting respiratory quotient (RQ), where higher doses of NPY injected into the PVN raised RQ, indicating a shift in fuel mix to carbohydrate oxidation (thus favoring fat storage) or an NPY-induced increase in lipogenesis. Although no clear effect of NPY was seen on energy expenditure in one study (18), recent work in mice has shown an NPY-induced reduction in energy expenditure as measured by calorimetry (33). Our evidence extends this concept by indicating that a central control element can modulate energy storage, LPL activity in white fat, in addition to modulating energy intake and expenditure.

The most likely mediator of the NPY/PVN stimulus to brown fat is the sympathetic nervous system. Evidence from nerve trunk firing rates shows that NPY administered into the cerebroventricular system induces reduction in sympathetic activity to brown adipose tissue (12). How the NPY/PVN signal reaches white fat is less clear. Important direct innervation of white fat is still in doubt. There are reports suggesting that NPY administration to hypothalamic sites can increase serum insulin concentrations (19). The data in this study and in our previous study (3) do not support an important effect of central NPY on insulin or glucose over the course of a 24-h study. However, it is still possible that the single time point at study end employed here is insufficient to detect a transient but important elevation of insulin provoked by the NPY/PVN stimulus. Studies showing the effect of NPY, usually given intracerebroventricularly, on insulin have generally measured at 2 h or less after NPY administration (1, 19, 33). Further, although one report (1) suggests that NPY administered into PVN can affect insulin, glucose, and glucagon over 10–30 min postinjection, another report places the potential central NPY effect on insulin in the nucleus of the solitary tract (11). Certainly none of the previous reports is comparable to these studies with respect to repetitive administration over 24 h. Whether transient elevations of insulin could contribute to the observed changes over 24 h is not known, and assessment will require further detailed studies. There are also reports of corticosterone elevation after NPY administration intracerebroventricularly (13, 31) and into hypothalamus (17), but we saw no such effect. Again, it is possible that transient effects on corticosterone could have been missed by our sampling procedure. It is unclear whether corticosterone could produce the observed effects on white fat LPL or brown fat UCP.

**Perspectives**

The role of the arcuate-NPY-PVN in the regulation of energy balance is gaining wide support. In addition to the anatomic and fasting-refeeding studies described above, it is known that NPY levels are increased in PVN of Zucker genetically obese rats (25), a model in which hyperphagia, reduced brown fat energy expenditure, and increased white fat LPL activity have all been described. The observation here that the NPY in the PVN can coordinate more than one component of energy metabolism to produce energy storage is particularly
significant when considered together with the many reports of responsiveness of NPY activity in the arcuate nucleus-PVN neural circuit to such perturbations of energy balance as fasting and feeding, diabetes, and genetic obesity. Taken together, the observations suggest the presence of a highly organized and comprehensive control element for energy metabolism that reaches far beyond simple appetite control.

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