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Publication Date
1998-09-01

DOI
10.1016/s0167-0115(98)00098-6

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Regulatory Peptides 75–76 (1998) 425–431

REGULATORY PEPTIDES

NPY-induced overfeeding suppresses hypothalamic NPY mRNA expression: potential roles of plasma insulin and leptin

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Received 16 December 1997; received in revised form 17 March 1998; accepted 23 March 1998

Abstract

To test the hypothesis that NPY-induced overfeeding activates compensatory responses that inhibit hypothalamic NPY gene expression, we investigated the effect of chronically administered neuropeptide Y (NPY) on plasma hormones involved in energy balance and on the level of mRNA for hypothalamic neuropeptides. After cannulation of the third cerebral ventricle, male Long-Evans rats received a 4.5-day intracerebroventricular (icv) infusion of either human NPY (12 μg per day), or synthetic cerebrospinal fluid (CSF). NPY-treated animals were either allowed ad libitum access to food or were pairfed to the intake of CSF-treated controls. In rats fed ad libitum, icv NPY induced significant increases in food intake (75%), body weight (9%), plasma insulin (150%) and plasma leptin levels (300%) as compared to the icv CSF group. Levels of plasma leptin, but not insulin, remained elevated in NPY-treated rats that were pairfed to the intake of the CSF group. NPY mRNA levels in the midregion of the arcuate nucleus (ARC) were reduced by 50% in NPY-treated rats that were allowed to overeat, but not in the pairfed group, as determined by in situ hybridization. In contrast, mRNA for corticotropin-releasing hormone (CRH) in the paraventricular nucleus (PVN) and proopiomelanocortin (POMC) in the rostral ARC were not significantly different among groups. These findings indicate that NPY-induced overfeeding suppresses ARC NPY mRNA expression, and that this effect unlikely to be mediated by a direct action of NPY, since it was abolished by limiting food intake in NPY-treated animals to that observed in controls. NPY-induced overfeeding was also associated with elevated plasma levels of leptin and insulin. The effect of these hormones to inhibit NPY gene expression may therefore have contributed to the decrease of NPY mRNA. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Neuropeptide; Obesity; Adipose tissue; Hypothalamus; Food intake

Numerous studies have found that NPY has a potent effect to increase food intake and body weight and can alter neuroendocrine function in rodents when injected chronically near hypothalamic areas that mediate energy homeostasis [1–9]. For example, when animals are allowed free access to food, chronic icv NPY administration increases levels of plasma corticosterone [3,9], plasma insulin [1,3–5,7,9] and leptin mRNA in inguinal white adipose tissue [7,9], while chronic infusion of NPY antibody lowers food intake [6]. Some of these responses (e.g. increased leptin mRNA expression [7] and glucocorticoids [1]) persist when hyperphagia is prevented by pairfeeding, suggesting that they result from an action of NPY independent of increased caloric intake.

Evidence for a physiological role for NPY in energy

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0167-0115/98/$ – see front matter © 1998 Elsevier Science B.V. All rights reserved.
PII: S0167-0115(98)00098-6

homeostasis derives from studies of factors regulating NPY gene expression in the arcuate nucleus (ARC) of the hypothalamus, and its release in the paraventricular nucleus (PVN). Several studies have found that conditions associated with weight loss, such as starvation [10–15] and uncontrolled diabetes [16,17], are associated with increased NPY gene expression and immunoreactivity. This response is implicated as a mediator of hyperphagia that promotes the recovery of lost weight.

Since NPY expression is increased with conditions of caloric deficit, one might expect that levels of NPY mRNA would be suppressed in animal models of overfeeding. While this hypothesis appears to be at odds with the coexistence of increased food intake with elevated ARC NPY gene expression in animals such as the leptin-deficient *ob/ob* mouse [18], this combination is expected if negative feedback signaling from adipose tissue is impaired. Insulin and leptin are hormones that circulate at levels proportionate to body adiposity and are implicated as negative feedback signals to the hypothalamus that participate in the inhibitory regulation of both food intake and ARC NPY gene expression. Thus, food intake and hypothalamic NPY levels are increased in conditions associated with deficiency of one or both of these hormones (e.g. starvation, uncontrolled diabetes, and genetic leptin deficiency in *ob/ob* mice), and replacement of the deficient hormone reduces both food intake and hypothalamic NPY production [18,19] and release [20]. Therefore, the hypothesis that insulin and leptin inhibit the NPY system during overfeeding provides a potential explanation for persistently elevated hypothalamic NPY synthesis in animals with defective leptin and/or insulin signaling, despite pronounced hyperphagia. In contrast, NPY mRNA expression in normal animals should be inhibited during NPY-induced overfeeding if it is accompanied by increased negative feedback from insulin and leptin.

We therefore hypothesized that NPY-induced overfeeding in normal animals would elevate plasma levels of insulin and leptin, and thereby suppress ARC expression of NPY mRNA, and indeed, preliminary support for this hypothesis was reported previously in abstract form [21]. In addition, we investigated the role of NPY, acting either locally in the hypothalamus or via modulation of plasma hormones, as a regulator of other hypothalamic neuropeptides involved in the regulation of energy balance. Specifically, we wished to determine if chronic infusion of NPY alters hypothalamic expression of mRNA encoding either proopiomelanocortin (POMC) or corticotropin-releasing hormone (CRH). This possibility is suggested by the observation that conditions associated with increased expression of NPY mRNA can coexist with decreased hypothalamic POMC mRNA or CRH mRNA levels in the rat [13,22,23].

To test these hypotheses, we studied the effects of a 4.5-day continuous intracerebroventricular NPY infusion on food intake, body weight, plasma hormone levels and expression of NPY, POMC and CRH mRNA in rats allowed ad libitum access to food. An NPY-infused group pairfed to CSF-infused controls was included in order to separate the local effects that exogenous NPY may have on hypothalamic neuropeptide levels from the response to NPY-induced hyperphagia and weight gain.

1. Materials and methods

1.1. Animals and procedures

Male Long-Evans rats (300–350 g) from the breeding colony maintained by the Department of Psychology at the University of Washington were housed individually in wire-mesh hanging cages in a temperature-controlled vivarium on a 12:12-h light:dark schedule. Unless otherwise specified, animals were given free access to pelleted rat chow (Harlan–Teklad, Madison, WI) and water at all times. All procedures were performed in accordance with institutional guidelines of the Animal Care Committee at the University of Washington.

1.1.1. Cannula placement

Rats were habituated with daily handling for one week prior to surgery. Animals ($n = 20$) were anesthetized by IP injection of ketamine/xylazine (60 mg/kg ketamine and 8 mg/kg xylazine) and a 21-gauge cannula (Plastics One, Roanoke, VA) was placed stereotaxically into the third ventricle using a previously described method [16,19]. Cannula placement was verified one week after surgery by icv injection (1 μl) of angiotensin II (10 ng/μl). Animals not consuming at least 8 ml water 30 min post injection were excluded ($n = 5$) [19]. Three weeks after cannula placement, rats were implanted subcutaneously with an osmotic minipump (Azlet model #2002, Palo Alto, CA) delivering either 1 μg/μl human neuropeptide Y (American Peptide Co., Sunnyvale, CA) in synthetic CSF vehicle at a rate of 0.5 μl/h, or vehicle alone, via a polyethylene catheter that was connected to the ventricular cannula.

1.1.2. Experimental design

Three weeks after cannula placement, animals were subdivided into three groups ($n = 5$/group) of equal body weight. One group received icv NPY infusion with ad libitum access to chow. The second group received icv synthetic CSF and was also fed ad libitum, while the third group received icv NPY infusion, but was permitted to eat only the amount of food consumed by the CSF control group. The amount of chow provided to each pairfed animal on each treatment day was equal to the measured amount of chow consumed by its CSF-treated partner during a previous 24-h period, and was divided into three equal portions administered at 0200, 1000 and 1800 h.

Animals received icv infusions of either NPY or CSF for four days, and were decapitated between 1000–1100 h on

the fifth day of infusion. Trunk blood, brains, livers and retroperitoneal fat pads were collected upon decapitation. Trunk blood was centrifuged and plasma was stored at −20°C. Retroperitoneal fat pads and livers were weighed and stored at −20°C.

1.2. Assays and data analyses

In situ hybridization (ISH) to NPY, CRH, and POMC mRNA. Brains for ISH were immediately frozen on crushed dry ice, and subsequently sectioned at 14 μm in a cryostat and mounted on RNAase-free slides. Hybridization for NPY and CRH mRNA was performed using ^{33}P-labeled antisense oligonucleotide probes based upon cDNA sequences of rat NPY or CRH genes, as described elsewhere [16,19] and a riboprobe complementary to rat POMC mRNA (a generous gift of Dr. Robert Steiner) was used for hybridization to POMC mRNA after labeling with ^{33}P, as previously described [24]. Slides for ISH to POMC mRNA were selected from the region of the ARC rostral to the ventromedial hypothalamic nucleus, and slides for NPY mRNA were selected from the midregion of the ARC. Slides for CRH mRNA were selected from the PVN. All slides were selected by an investigator blinded to the treatment group. Labeled slides were washed under high stringency conditions and opposed to X-ray film to generate autoradiographs, which were analyzed by computer densitometry. Using a standard curve, autoradiographic optical density and hybridization area were determined on 6–8 sections/rat using the MCID computer densitometry system (Imaging Research, St. Catherine's, Ontario, Canada). The product of hybridization area (pixels) and density (μ Ci/pixel) was used as an index of overall neuropeptide mRNA levels [16,18,24,25].

1.2.1. Plasma assays

Radioimmunoassays were used to measure plasma levels of corticosterone and immunoreactive insulin (IRI) as previously described [19,26]. Plasma glucose was determined by the glucose oxidase method (Beckman Instruments, Brea, CA). Plasma leptin levels were determined using a rat-specific antibody (Linco, St. Louis, MO).

1.2.2. Statistical analyses

All statistical analyses were carried out using Prism 2.01 (GraphPad Software, Inc., San Diego, CA) statistical software. Data are presented as group mean values (±SEM). Comparisons were performed with one-way analysis of variance with Neuman-Keuls post hoc test. A P value ≤ 0.05 between group mean values was considered statistically significant.

2. Results

2.1. Food intake

As summarized in Table 1, food intake was significantly greater among NPY-infused rats fed ad libitum ($P < 0.05$) than in the CSF-infused group on the first day of infusion. This effect increased with each successive day until the time of sacrifice ($P < 0.002$). Cumulative food intake over the 4.5-day study period was 75% greater in the NPY-infused group than in ad libitum fed controls ($P < 0.001$). As planned, food intake of the NPY-treated pairfed group was matched precisely to that of the CSF group, and was significantly less than that of NPY-treated rats provided ad libitum access to food.

2.2. Body weight

As summarized in Table 1, mean body weight at baseline was similar among the three groups of rats. When expressed as a percentage of initial body weight, weight at the time of sacrifice was increased by 8.7%±0.7% in NPY-treated rats fed ad libitum as compared to the CSF-infused ad libitum-fed animals and 10.2%±0.7% as compared to the NPY-infused pairfed animals ($P < 0.001$ for both comparisons). Body weight was not significantly different between NPY-infused pairfed rats and the CSF-infused controls. Food in the gastrointestinal tract at sacrifice was also significantly greater in the NPY ad libitum group as compared to both of the other groups (+15.5%±4.7% vs CSF and +10.7%±2.9% vs NPY pairfed; $P < 0.05$ for both comparisons).

When expressed as a percentage of total body weight minus the weight of food in the gastrointestinal tract at sacrifice, liver and retroperitoneal fat pad weights in the NPY-infused ad libitum group were increased over CSF-infused and pairfed groups by 30% and 40%, respectively, although this effect reached statistical significance only for liver weight (Table 1). The weight of both liver and

Table 1
For NPY vs CSF and NPY pairfed

	CSF	NPY	NPY pairfed
Cumulative food intake over 4.5 days, g	115.30±5.99	201.60±16.00[b]	115.30±5.99
Body weight at sacrifice, g	408.34±14.74	448.04±20.20[a]	410.20±14.87
Delta BW/initial BW%	−0.33±0.24	8.34±0.76[c]	−1.83±0.59
Retroperitoneal fat pads/BW%	1.31±0.12	1.88±0.21	1.35±0.12
Liver weight/BW%	3.67±0.16	5.07±0.46[a]	3.88±0.11

[a] $P < 0.05$, [b] $P < 0.001$, [c] $P < 0.0001$.

retroperitoneal fat pads of CSF-infused animals were not different from those of NPY-treated pairfed controls.

2.3. Plasma values

Plasma leptin was increased by 300% in the NPY infused ad libitum group as compared to the CSF group ($P < 0.001$) and was 60% higher in the NPY infused ad libitum group as compared to the NPY pairfed group ($P < 0.01$) (Fig. 1A). Differences in leptin levels were also significant between CSF and NPY pairfed groups, with the NPY-infused pairfed group having a nearly 200% elevation over the CSF group ($P < 0.01$) (Fig. 1A).

Plasma corticosterone levels were increased by 90% in the NPY infused ad libitum group as compared to the CSF-infused group (105.6ng/ml±37.51 vs 55.76ng/ml±33.71), and by 180% in the NPY pairfed group (160.46ng/ml±11.94) (Fig. 1B), although neither difference reached statistical significance. Plasma insulin levels were 150% higher in the NPY ad libitum group than in either the CSF or the NPY pairfed group ($P < 0.01$) (Fig. 1C). In contrast to the effect of icv NPY to increase leptin levels in pairfed rats, however, insulin levels were not different between pairfed and CSF-treated controls. Plasma glucose levels were not significantly different between groups (Fig. 1D).

2.4. In situ hybridization

As depicted in Fig. 2A, ARC NPY mRNA levels were significantly lower in the NPY ad libitum group as compared to the levels seen in either the CSF or NPY pairfed groups (−55.3 and −45.2%, respectively; $P < 0.05$ for both comparisons). PVN CRH mRNA and ARC POMC mRNA also tended to be decreased in the NPY ad libitum group as compared to the CSF and NPY pairfed groups, although these differences did not reach statistical significance (Fig. 2B, C). The only statistically significant effect of icv NPY on hypothalamic mRNA levels measured in this study, therefore, was on expression of NPY mRNA. This effect, however, was prevented by limiting food intake to that of the CSF-treated controls.

3. Discussion

Conditions associated with weight loss increase hypothalamic NPY production and release [10–16], and this response is implicated in the hyperphagic response that

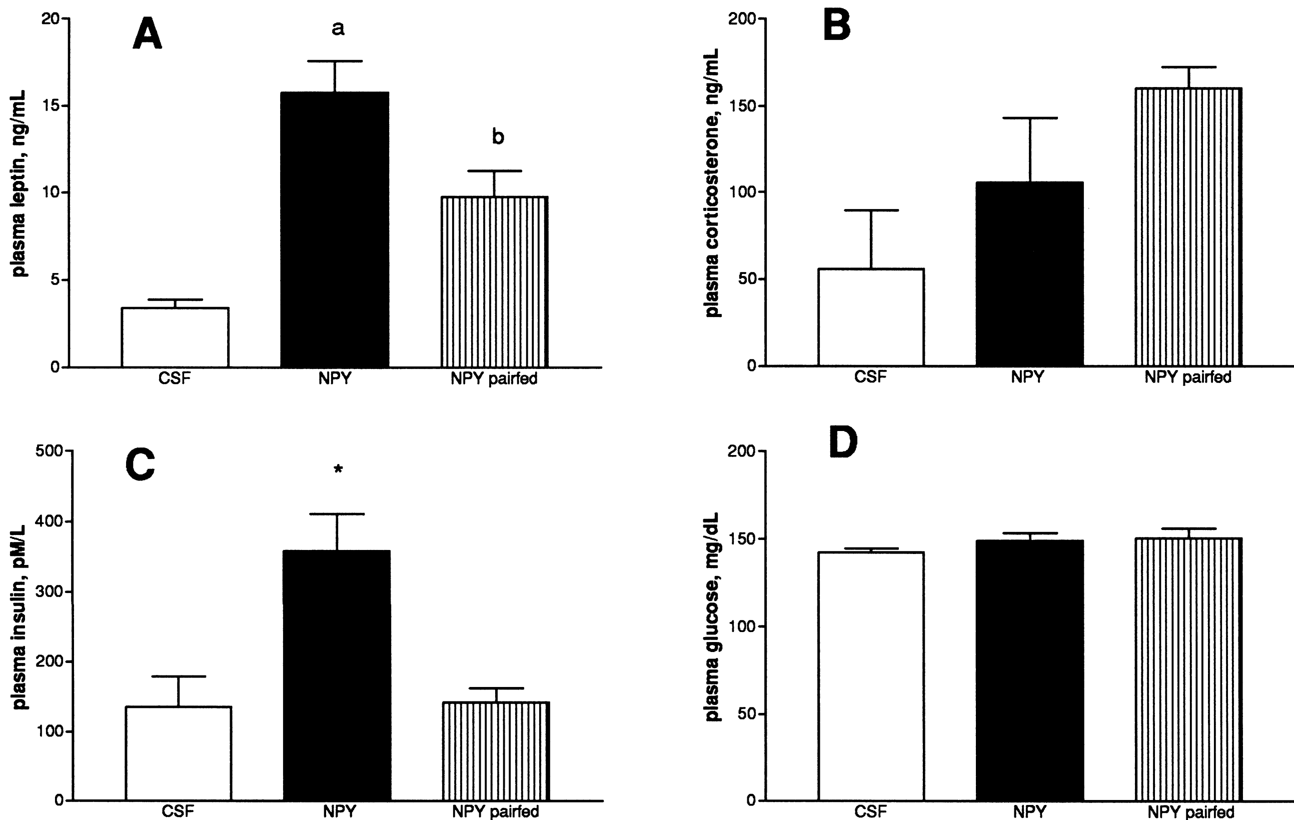

Fig. 1. Effect of chronic NPY infusion on plasma levels. 1A. Plasma leptin levels, [a]$P < 0.001$ vs CSF controls, [b]$P < 0.01$ vs CSF controls. 1B. Plasma corticosterone levels. 1C. Plasma insulin levels, *$P < 0.01$ vs CSF and NPY pairfed controls. 1D. Plasma glucose levels. For all figures, the NPY ad libitum fed group is represented by solid bars, the CSF group by open bars, and the NPY pairfed group by striped bars ($n = 5$/group).

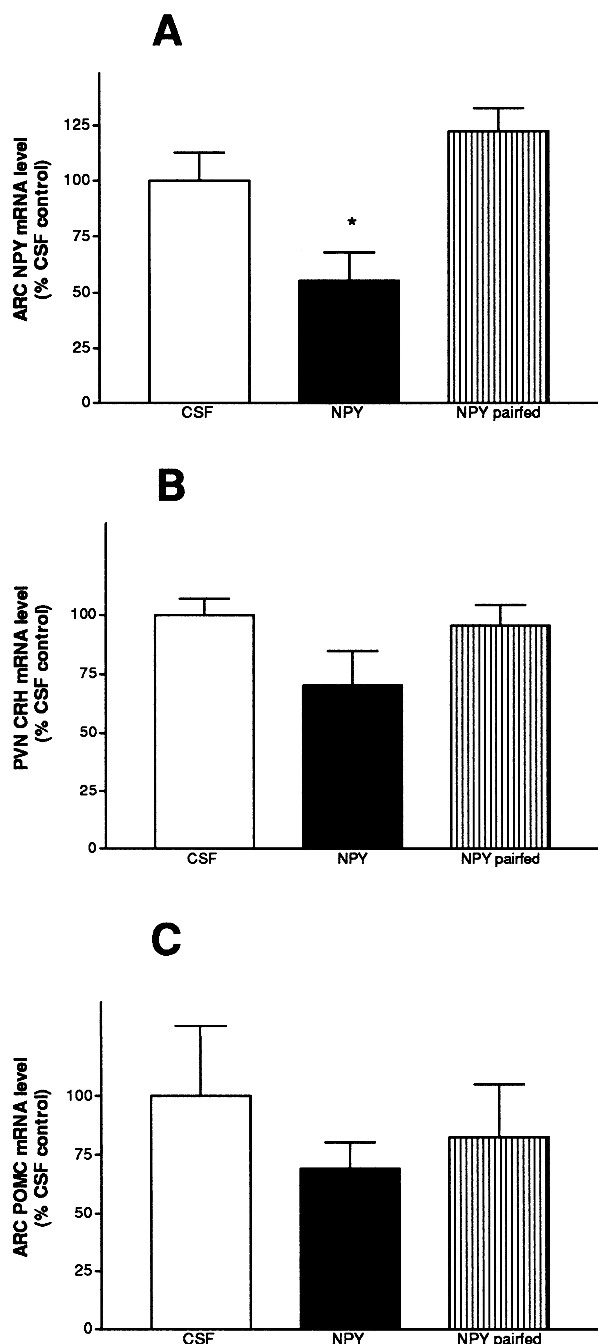

Fig. 2. Effect of chronic NPY infusion on hypothalamic neuropeptide expression determined by in situ hybridization. 2A. Levels of NPY mRNA in the midregion of the arcuate nucleus, $*P<0.05$ vs CSF and NPY pairfed controls. 2B. Levels of CRH mRNA in the PVN. 2C. Levels of POMC mRNA in the region of the arcuate nucleus rostral to the ventromedial hypothalamic nucleus. For all figures, the NPY ad libitum fed group is represented by solid bars, the CSF group by open bars, and the NPY pairfed group by striped bars ($n=5$/group except for 2A and 2C, where $n=4$ for NPY pairfed).

replenishes depleted fuel stores. Overfeeding might therefore be predicted to suppress the NPY system, especially if it increases levels of hormones such as insulin and leptin that inhibit ARC NPY gene expression. To test this hypothesis, we performed chronic icv NPY administration to induce an increase of food intake and body weight, which thereby increased plasma levels of insulin and leptin. We found that rats infused with NPY for 4.5 days had a 55.3% reduction of NPY mRNA expression in the midregion of the ARC as compared to CSF-infused controls, and that this response was accompanied by significant elevations of plasma leptin and insulin.

The mechanism by which hypothalamic NPY expression was downregulated may have involved overfeeding-induced elevation of plasma leptin and insulin. Alternatively, exogenous administration of NPY may have acted locally in the brain to suppress ARC NPY mRNA expression. To distinguish between these two possibilities, we controlled for overfeeding and its hormonal sequelae by including a group of NPY-infused rats with food intake yoked to ad libitum-fed, CSF-infused animals. Since ARC NPY mRNA was not suppressed in this pairfed group, our results support the hypothesis that it is NPY-induced overfeeding, rather than a direct action of NPY in the brain, that is responsible for suppression of NPY mRNA. Since several endpoints of our study are sensitive to the size and timing of meals (e.g. plasma insulin and glucose levels), it is possible that they were influenced by the pairfeeding regimen itself. Our observation that significant differences were not detected between the NPY-pairfed and the CSF control groups in any endpoint except for leptin levels, however, suggests that an independent effect of the pairfeeding regimen is unlikely to explain our results.

Plasma insulin levels were elevated by 150% and plasma leptin levels by 300% in the NPY-infused ad libitum group relative to the CSF-infused controls. Since neither insulin levels nor NPY mRNA levels were elevated in the pairfed group relative to the CSF controls, while leptin levels remained significantly elevated above CSF controls, our results suggest that the combination of increased leptin and insulin signaling may be required for overfeeding-induced suppression of ARC NPY mRNA expression.

Previous studies [27,28] have found that plasma leptin levels vary in proportion to fat stores and caloric intake. The effect of NPY-induced overfeeding to increase leptin levels was therefore expected. However, the pairfed group also had a marked elevation in plasma leptin levels in response to icv NPY infusion despite the fact that body composition and food intake were not different from those of the CSF group. That leptin levels were elevated in the face of maintained body weight and fat stores is a paradoxical finding that suggests a direct action of NPY in the brain to stimulate adipocyte leptin secretion. A previous study asserted that insulin may mediate this upregulation [7], yet our findings indicate that leptin levels increased independent of hyperinsulinemia, and recent evidence indicates that adipocyte glucose metabolism may mediate this action [29]. An alternative mechanism to explain this NPY effect involves an inhibition of sympa-

thetic outflow induced by NPY [30]. Conditions that increase sympathetic nervous system outflow lower leptin levels [31], and NPY reduces SNS outflow. Therefore, it is possible that NPY may have acted in the central nervous system to elevate leptin levels via diminished SNS tone to the adipocyte. Inhibition by NPY of the mechanisms responsible for leptin clearance from the circulation provides a third potential explanation for this result. Further studies are warranted to test these hypotheses.

Recently it was demonstrated in an overfeeding paradigm that a 25% increase in insulin levels and a 5% weight gain induced by involuntary overfeeding in rats did not change ARC NPY mRNA expression [32]. In that study, caloric intake was incrementally increased by gastrostomy feeding of a condensed milk formula over 10 days, and resulted in a marked suppression of spontaneous food intake after body weight was significantly elevated. The rate of weight gain (0.5% per day) in that study, however, was much lower than in the present study (2% per day). The significant suppression in ARC NPY levels seen in the present study may therefore reflect the relatively greater caloric excess and elevation of insulin and leptin levels induced by central NPY administration as compared to gastrostomy feeding.

The response of the hypothalamic CRH system also reveals differences between the response to overfeeding induced by NPY and that induced by gastrostomy feeding. In the previous study [32], PVN CRH mRNA was elevated to 150% of control levels in rats overfed by gastrostomy, consistent with the hypothesis that activation of the CRH system is a component of the regulatory response to involuntary overfeeding. In contrast, we report here no change in PVN CRH mRNA levels in animals with NPY-induced overfeeding. Similarly, POMC mRNA levels in the rostral ARC were not significantly changed in NPY-infused, ad libitum fed animals as compared to controls. While these data suggest that central NPY administration does not alter hypothalamic expression of CRH or POMC genes, it is also possible that NPY elicited multiple, offsetting effects on these neuropeptide systems. For example, since leptin has been shown to increase hypothalamic expression of both CRH and POMC mRNA [24,25], an increase in these mRNA species might have been expected in NPY-infused rats, since leptin levels were elevated. Since this was not observed, it is conceivable that NPY itself, either directly or indirectly, exerted effects on CRH and POMC neurons that offset the effect of elevated leptin levels. Accurate interpretation of CRH and POMC mRNA data in our study therefore may require experiments with a larger sample size that are designed to control for these potentially competing effects elicited by icv NPY.

In conclusion, our findings indicate that NPY-induced overfeeding suppresses NPY mRNA expression in the midregion of the ARC. We hypothesize that this effect was mediated via the additive effects of elevated insulin and leptin signaling to inhibit NPY gene expression, since prevention of the NPY-induced increase in food intake by pairfeeding blocked the suppression of NPY mRNA expression and lowered plasma leptin and insulin levels. In addition, our data suggest that NPY can activate a central nervous system pathway that increases leptin secretion, since plasma leptin levels were elevated in NPY-infused pairfed animals in the absence of any increase in body weight. The concept of a neuronal system for regulating leptin secretion in vivo warrants further study.

Acknowledgements

This work was supported by grants from the National Institutes of Health (DK12829, DK52789, NS32273, DK50129, DK35747, DK17844 and DK54080), the Diabetes Endocrinology Research Center, the Clinical Nutrition Research Unit at the University of Washington, and the VA Merit Review Program. Technical assistance for in situ hybridization was provided by Vicki Hoagland and Zoe Jonak, and the corticosterone assay was performed by Elizabeth Colasurdo and Shannon Reed. The riboprobe for POMC mRNA was generously provided by Dr. Robert Steiner.

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