

# Regulation of Hypothalamic Neuropeptide-Y Neurons by Growth Hormone in the Rat\*

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## ABSTRACT

GH is thought to exert a short-loop feedback action on the hypothalamic somatostatin- and GH-releasing hormone (GHRH)-containing neurons. The direct actions of GH are mediated through GH receptors. In the male rat, few GHRH-containing neurons in the arcuate nucleus (ARC) appear to express the GH receptor messenger RNA (mRNA); however, some unidentified neurons near GHRH neurons do. Recent evidence suggests that neuropeptide-Y (NPY)-containing neurons, which are located near GHRH neurons in the ARC, are targets for GH action because treatment of rats with GH induces *c-fos* expression in these cells. We conducted two experiments to test the hypothesis that GH acts on NPY neurons in the ARC. First, we performed double-label *in situ* hybridization to determine whether NPY neurons in the ARC express GH receptor mRNA. Second, we

investigated the possibility that GH regulates NPY mRNA expression by using *in situ* hybridization to compare ARC NPY mRNA levels among groups of normal ( $n = 7$ ), hypophysectomized ( $n = 7$ ), and hypophysectomized/rGH-treated (1.5 mg rat GH over 3 days;  $n = 6$ ) rats. We found that most of the NPY-containing neurons in the ARC expressed GH receptor mRNA, whereas hypothalamic NPY neurons residing outside of the ARC did not. Furthermore, hypophysectomy significantly decreased NPY mRNA levels, and GH treatment restored the levels to those of the intact animals. We conclude that GH regulates the activity of NPY neurons in the ARC by a direct action on GH receptors that are expressed by NPY neurons. Whether the action of GH on NPY neurons in the ARC is related to the feedback control of GH secretion or some other physiological function remains to be determined. (*Endocrinology* 137: 1319–1325, 1996)

GH IS SECRETED in a pulsatile manner, and its patterning is dependent upon the reciprocal action of somatostatin (SS) and GH-releasing hormone (GHRH) on pituitary somatotropes (1). GHRH is released from neurons whose cell bodies reside in the arcuate nucleus (ARC), and it stimulates the synthesis and secretion of GH from the pituitary (2–4). SS is released from neurons whose cell bodies reside primarily in the periventricular nucleus (PeN), and it inhibits the secretion of GH (5, 6). GH participates in the homeostatic control of its own secretion through a mechanism involving short-loop feedback regulation on the synthesis and release of SS and GHRH. Evidence suggests that GH stimulates the synthesis and secretion of SS from neurons in the PeN and inhibits the synthesis and secretion of GHRH from neurons in the ARC (7–10).

The direct action of GH is mediated by GH receptors. GH appears to act directly on hypothalamic SS neurons because GH receptor messenger RNA (mRNA) is expressed in the majority of SS neurons in the PeN and the paraventricular nucleus (PVN) (11); however, the effect of GH on GHRH neurons is most likely indirect because few GHRH neurons in the ARC appear to express the GH receptor mRNA (12). A population of cells in the ARC do appear to express GH receptor mRNA, but their identity has not been revealed (12). Neuropeptide-Y (NPY) neurons reside in close proximity to GHRH neurons in the ARC, and GH induces the rapid ex-

pression of the early response gene, *c-fos*, in these NPY neurons (13). These observations suggest that NPY neurons are a plausible target for the direct action of GH in the ARC. If this were the case, then NPY neurons in the ARC should express the mRNA for the GH receptor. We tested this hypothesis by examining the coexpression of GH receptor mRNA and NPY mRNA in the hypothalamus by double-label *in situ* hybridization.

The induction of *c-fos* mRNA in NPY neurons by GH and the presence in the NPY promoter region of an activator protein 1 (AP-1) regulatory element (14), which interacts with Fos, the gene product of *c-fos*, suggests that NPY gene expression is regulated by GH. We tested the hypothesis that GH regulates the expression of NPY mRNA in the ARC by comparing cellular levels of NPY mRNA among groups of rats in which plasma levels of GH had been experimentally altered by hypophysectomy and GH replacement.

## Materials and Methods

### Exp 1

Two adult male rats were killed, and brains were collected and sectioned. Tissue sections that included the ARC were processed by double-label *in situ* hybridization and analyzed for double-labeling of GH receptor mRNA and NPY mRNA.

### Exp 2

Hypophysectomized (hypox) adult male rats were treated with either rat GH (rGH, 1.5 mg;  $n = 6$ ) or vehicle ( $n = 7$ ) over a 3-day period. Normal (intact) adult male rats received only the vehicle for the same length of time ( $n = 7$ ). At the end of the treatment period, the rats were killed and their brains and trunk blood were collected. The brains were

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processed for NPY mRNA by single-label *in situ* hybridization, and serum levels of rGH were measured by RIA.

### Animals and accommodations

For Exp 1, adult male Sprague-Dawley rats (70 days old) were purchased from Simonsen Laboratories (Gilroy, CA). For Exp 2, intact and hypox adult male Sprague-Dawley rats (70 days old) were purchased from Charles River Laboratories (Wilmington, MA). All animals were housed in group cages (4 animals/cage) in the animal-care facilities of the University of Washington Department of Comparative Medicine. The facilities were maintained at a constant temperature and a 14-h light/10-h dark cycle with lights on at 0700 h and off at 2100 h. Rat chow and water was available *ad libitum*, and hypox animals were given water containing 35 mM NaCl, 1.1 mM KCl, 0.24 mM CaCl<sub>2</sub>, and 0.18 mM MgCl<sub>2</sub>. To permit the animals to acclimatize to their new environment, the experiments were performed 2 weeks after their arrival from the supplier. All animal procedures were approved by the Animal Care Committee of the School of Medicine at the University of Washington.

### Surgeries and hormone treatment (Exp 2)

The animals were anesthetized by ether inhalation between 1100 h and 1200 h while osmotic minipumps (Model 1003D, Alzet, Palo Alto, CA) were implanted into the interscapular sc space. These minipumps dispensed over a period of 3 days a solution containing either vehicle (0.03 M NaHCO<sub>3</sub>, 0.15 M NaCl, and 1% BSA) or rGH (1.5 mg in the vehicle).

### Tissue preparation

Animals in both experiments were killed by rapid decapitation between 1100 h and 1200 h. Brains were removed, frozen on dry ice, and stored at -80 C. Using the rat atlas of Paxinos and Watson as an anatomical guide (15), we collected coronal brain slices (for Exp 1, 15  $\mu$ m; for Exp 2, 20  $\mu$ m) with a cryostat, beginning rostrally at the decussation of the anterior commissure and continuing caudally through the ARC. The sections were thaw-mounted onto four sets of Superfrost/Plus Microscope Slides (Fisher Scientific, Pittsburgh, PA), thereby placing every fourth slice into a given set. Slides were stored in air-tight boxes at -80 C until hybridization histochemistry was conducted.

### Complementary DNA (cDNA) probes

The plasmid pG0.9, containing a 960 bp *Bgl*III fragment of the rat GH receptor cDNA cloned into the *Bam*HI site of the vector pT7T318U, was kindly provided by Dr. L. S. Mathews and Professor G. Norstedt (16). This cDNA fragment spans the extracellular domain of the GH receptor and recognizes both alternatively spliced products of the GH receptor gene, *i.e.* the membrane-spanning receptor and the circulating binding protein. The rat pre-pro-NPY cDNA probe was generously provided by Dr. Steven Sabol (17). The 511 bp pre-pro-NPY cDNA fragment, containing the full-length sequence, was inserted into the *Eco*RI site of the Bluescribe M13(-) vector.

### Probe preparation

For *in vitro* antisense GH receptor cRNA and NPY cRNA probe syntheses, the linearized plasmid DNA was transcribed with [<sup>35</sup>S]  $\alpha$ -thio-UTP (New England Nuclear Research Products, Boston, MA) at a concentration of 50  $\mu$ M, using T<sub>7</sub> and T<sub>3</sub> RNA polymerase, respectively. Residual DNA was removed by digestion with DNase (Promega Biotec, Madison, WI), and the cRNA probes were purified on a Nensorb column (New England Nuclear-DuPont, Wilmington, DE). The identity and integrity of both transcripts were verified by polyacrylamide gel electrophoresis against known standards.

Digoxigenin-labeled cRNA probe for NPY mRNA was synthesized from linearized plasmid DNA, transcribed *in vitro* from a digoxigenin labeling mixture containing 3.5 mM digoxigenin UTP, 6.5 mM unlabeled UTP, and 10 mM GTP, ATP, and CTP at pH 6.5 (Boehringer Mannheim, Indianapolis, IN), and T<sub>3</sub> RNA polymerase. Residual DNA was digested

with DNase, and the cRNA probe was purified on a G-50 Sephadex column (Boehringer Mannheim, Indianapolis, IN).

### Single-label *in situ* hybridization histochemistry for NPY mRNA

Using the <sup>35</sup>S-labeled riboprobe complementary to NPY mRNA, we performed single-label *in situ* hybridization as previously described (18). In brief, after fixation in 4% paraformaldehyde, tissue sections were rinsed in phosphate buffer and pretreated with 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8) for 10 min. The slides were rinsed in 2  $\times$  SSC (1  $\times$  SSC = 150 mM NaCl and 15 mM Na citrate), dehydrated through a series of alcohols, delipidated in chloroform, partially rehydrated in alcohols, and air-dried. The probe was applied at a concentration of 2 pmol/ml in a hybridization buffer containing 52% formamide, 10% dextran sulfate, 0.3 M NaCl, 8 mM Tris, 0.8 mM EDTA, 0.02% BSA, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 200 mM dithiothreitol, and 0.5 mg/ml yeast transfer RNA. The slide was covered with a silanized coverslip, and the sections were incubated overnight in moist chambers at 52 C. On the following day, the coverslips were rinsed off in 2  $\times$  SSC. After treatment with RNase-A and a series of washes in SSC of increasing stringencies, the slides were dehydrated in alcohols and air-dried. The slides were dipped in Kodak NTB-2 emulsion (Eastman Kodak, Rochester, NY), heated to 45 C in a water bath, and diluted 1:1 with 600 mM ammonium acetate. They were allowed to air-dry for 45 min and placed in light-tight boxes containing small tubes of desiccant. They were exposed for 7 days at 4 C. Slides were then developed in Kodak D-19 (diluted 1:1 with distilled water), fixed with Kodak fixer, and rinsed in water. Sections were counterstained with cresyl violet, dehydrated through alcohols, and cleared in Hemo-De (Fisher Scientific, Springfield, NJ), and coverslips were then applied.

### Double-label *in situ* hybridization for NPY mRNA and GH receptor mRNA

We performed double-label *in situ* hybridization to identify cells containing both NPY mRNA and GH receptor mRNA following a protocol that was similar to that described for the single-label *in situ* hybridization and similar to previously described double-label *in situ* hybridizations (19), with modification. Briefly, before the hybridization procedure, both the <sup>35</sup>S-labeled GH receptor cRNA probe and the digoxigenin-labeled NPY cRNA probe were heat denatured and diluted to a concentration of approximately 2 pmol/ml in the hybridization buffer. Because the exact yield of the transcription reaction with digoxigenin-UTP for the NPY cRNA probe could not be derived, a test *in situ* hybridization was performed to determine empirically the optimal concentration for this probe. After the second day of stringent washes, the slides were placed in a blocking buffer containing 2  $\times$  SSC, 0.05% Triton X-100, and 2% normal sheep serum for 60 min. The slides were washed in buffer 1 (100 mM Tris-HCl, pH 7.5 and 150 mM NaCl) for 30 min and then incubated for 3 h at 37 C with antidigoxigenin antibody conjugated to alkaline phosphatase (Boehringer Mannheim Biochemica, Indianapolis, IN; 1:1000 in buffer 1 containing 1% normal sheep serum and 0.3% Triton X-100). The slides were washed in buffer 1 again and rinsed in buffer 2 (100 mM Tris-HCl, 100 mM NaCl, 50 mM MgCl<sub>2</sub>, pH 9.5) for 10 min. The slides were then incubated in buffer 2 with 340  $\mu$ g/ml nitroblue-tetrazolium-chloride (Sigma, St. Louis, MO) and 175  $\mu$ g/ml 5-bromo-4-chloro-3-indolyl-phosphate (Sigma), and 240  $\mu$ g/ml levamisole in a light-tight box for 3 h at 37 C. The reaction was stopped by rinsing slides in 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA. The slides were placed in 70% ethanol for 15 sec and then air-dried. Before being emulsion-coated, the slides were dipped in 3% parlodion (Fisher Scientific, Fair Lawn, NJ), which was dissolved in isoamyl acetate. Slides were placed in light-tight boxes containing small tubes of desiccant and were exposed for 21 days at 4 C. Slides were then developed in Kodak D-19 (diluted 1:1 with distilled water), fixed with Kodak fixer, and rinsed in water. Sections were dehydrated through alcohols, cleared in Hemo-De (Fisher Scientific, Springfield, NJ), and coverslips were applied.

### Control experiments

Two control experiments were performed to assess the specificity of binding of the NPY cRNA probe to the tissue. These control experiments

have been performed in our lab previously for the GH receptor probe (11). First, an  $^{35}\text{S}$ -labeled sense probe was placed on tissue to establish that such a probe would not produce grain clusters. Second, 100-fold greater concentration of unlabeled antisense probe in the presence of  $^{35}\text{S}$ -labeled antisense probe (at a concentration of 2 pmol/ml) was placed on the tissue to show that the unlabeled probe would displace the hot label. The tissue was processed for *in situ* hybridization histochemistry as previously described for single-label hybridization. Both experiments resulted in complete absence of autoradiographic grain clusters over cells, which we interpreted as evidence that the  $^{35}\text{S}$ -labeled probe was binding to a specific sequence in the NPY mRNA (data not shown).

A third control experiment was performed to determine the specificity and extent of labeling of the digoxigenin-labeled NPY riboprobe. A double-label *in situ* hybridization was performed with both an  $^{35}\text{S}$ -labeled and a digoxigenin-labeled NPY cRNA probe on the same tissue section. We found that more than 99% of the cells labeled in the ARC had both autoradiographic silver grains and chromagen present (data not shown).

### Image analysis

*Exp 1.* Forty-five tissue sections per animal equally spaced from the PeN through the ARC were examined for the presence of double-labeled cells by visual inspection. Sections containing the ARC were further analyzed by an automated image processing system to estimate the percentage of NPY cells that express GH receptor mRNA. The image processing system consisted of a Pixel Grabber video acquisition board (Perceptics Corp., Knoxville, TN) attached to a Macintosh IIfx computer (Apple Computer Corporation, Cupertino, CA). The tissue sections were viewed under a Zeiss Axioskop microscope (Zeiss, New York, NY) equipped with a  $40\times$  epi-illumination darkfield objective. Video images were obtained by a Dage model 65 camera (Dage-MTI, Inc., Michigan City, IN) attached to the microscope.

For each section, two fields on each side of the third ventricle were analyzed. First, each field was visualized under brightfield illumination, and the boundaries of the NPY cRNA/digoxigenin-containing cell bodies were outlined. Next, the illumination was switched to darkfield, and silver grains were counted over the outlined cells and over the area surrounding the cells (the background area). As previously described, silver grain centers were identified by local light-intensity peaks above a threshold that is determined by the gray level histogram over the background area (11). Approximately 10 cells per section in the ARC were analyzed in this manner, and approximately 5 randomly chosen NPY cells per tissue section were analyzed in the cortex.

Two approaches were used to estimate the percent of NPY neurons expressing GH receptor mRNA in the ARC. The first was to analyze the *signal to background ratio* by calculating the ratio of the number of grains appearing over the NPY neurons in the ARC to the number of grains that would have occurred over an equivalent area in the background (*i.e.* background counts). Only cells having a ratio that was as high as or higher than a threshold value were considered double-labeled. Several different threshold values ranging from 1 to 5 were used in this approach. The second approach was to analyze the *signal density* over NPY cells by subtracting background counts from the specific number of grains counted over NPY neurons and dividing this value by the cross-sectional area of the neuron (in pixels). Again, only cells having specific grain densities as high as or higher than a threshold value were considered double-labeled. Threshold values ranging from 2–16 grains/1000 pixels were used in this instance to calculate the percent of NPY neurons in the ARC that express GH receptor mRNA. To assess the false-positive rate (*i.e.* when cells are counted as double-labeled but do not actually express the GH receptor mRNA) associated with a particular threshold, this same process was applied to NPY neurons in the cortex, where NPY cells are not thought to express the GH receptor mRNA.

*Exp 2.* According to the stereotaxic atlas of Paxinos and Watson (15), sections through the ARC (from plates 29–34) of each animal were atlas-matched between and within groups. Slides were analyzed for the  $^{35}\text{S}$ -labeled NPY cRNA probe with a computerized image analysis system (MCID, Imaging Research, Ontario, Canada). Video images were obtained by a Dage model CCD-72 camera (Dage-MTI, Inc., Michigan City, IN) attached to a Nikon Optiphot II microscope (Melville, NY)

equipped with a  $4\times$  objective and darkfield condenser. A template, shown in Fig. 1, was developed for the ARC, based on the general distribution of NPY cRNA signal in the mid region of the ARC at the level of the dorsomedial nucleus (DMN) of the hypothalamus (plate 31). This template was positioned over each section with the third ventricle and the base of the forebrain as borders. The average gray level of the area enclosed by the template was determined for each section and then averaged across all slides for each animal. This reading was used as an estimate of the intensity of cell labeling.

### RIA for rGH

Serum was collected from blood samples and frozen at  $-20\text{ C}$  until assayed. Serum levels of rGH were measured by a double-antibody RIA under the auspices of the RIA Core of the Population Center for Research in Reproduction at the University of Washington (Dr. William Bremner, Director) with reagents provided by National Pituitary Agency. The antibody used was anti-rat GH-RIA-5 from NIDDK, Bethesda, MD. The  $^{125}\text{I}$ -rGH was purchased from Corning Hazelton, Inc. (Vienna, VA). Final values were expressed in terms of nanogram equivalents of NIDDK rat GH-RP-2 per ml of serum. The intraassay coefficient of variation was 6.6%, and the minimum amount of rGH detectable by the assay was 0.63 ng/ml.

### Statistical analysis

Unless otherwise noted, all data are presented as mean  $\pm$  SEM. For Exp 2,  $n$  = the number of animals within a group, and this was the “ $n$ ” used in the data analysis. The statistical comparison of signal levels among groups was performed by one-way ANOVA. Fisher’s protected least significant difference test was used to identify specific differences between groups. A probability of 0.05 or less was considered significant for rejecting the null hypothesis that experimental and control values did not differ.

## Results

### *Exp 1: colocalization of GH receptor mRNA and NPY mRNA*

Numerous NPY mRNA-positive neurons in the PeN, PVN, DMN, and ARC were identified by the presence of a cytoplasmic purple reaction product in the cell. The silver grains, representing the presence of GH receptor mRNA, were diffusely distributed in the ARC and appeared as distinct clusters in the PeN, PVN, and DMN. By visual inspection

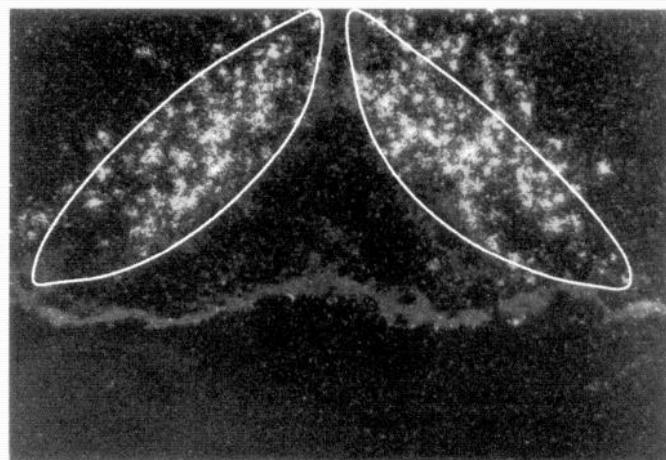


FIG. 1. Schematic of the template (white line) used in Exp 2 to measure NPY mRNA signal levels. As described in *Materials and Methods*, a computerized image analysis system was used to determine the average gray level of the area enclosed by the template.

tion, the majority of the NPY mRNA-containing neurons in the ARC appeared to express GH receptor mRNA (Fig. 2), whereas no NPY neurons residing within the PeN, PVN, or DMN appeared to express GH receptor mRNA (not shown).

A total of 164 digoxigenin-positive neurons in the ARC of one rat and 166 in the ARC of another rat were analyzed by automated image analysis. A summary of the *signal to background ratio analysis* for these cells is shown in Table 1. Because the selection of a criterion used to define a double-labeled cell is relatively arbitrary, results are shown for ratios ranging from 1–5. The results from both animals agreed within 4 percentage points for all 5 ratios. Overall, 100% of the NPY neurons in the ARC had signal levels that were at least as high as the background, and 61% had signal levels that were at least five times as high as the background. Whereas a ratio of 1:1 produces an estimate of coexpression that is too liberal and a ratio of 5:1 produces an estimate that is probably too conservative, selection of an intermediate ratio of 3:1 produced an estimate that approximately 92% of the NPY neurons in the ARC express GH receptor mRNA.

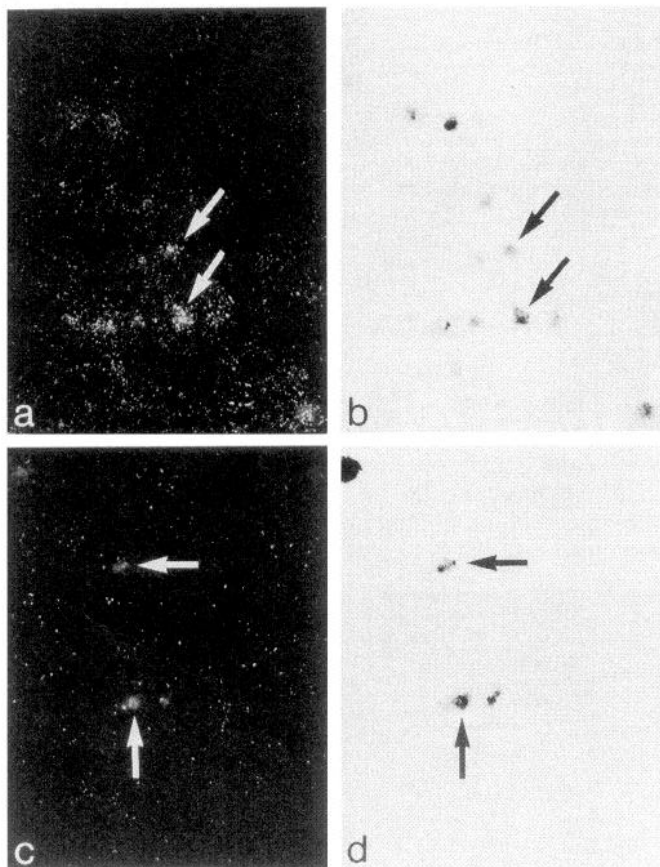


FIG. 2. Photomicrographs of neurons containing NPY mRNA and GH receptor mRNA in the ARC (a and b) and cortex (c and d). In each area, the same set of neurons were photographed under darkfield (a and c) and brightfield (b and d) illumination. NPY mRNA-containing neurons are filled with a dark precipitate that can be seen in brightfield. In darkfield, white-silver grains appear clustered over cells that contain GH receptor mRNA. *Black arrows* in b and d point to examples of NPY mRNA-containing neurons; *white arrows* in a and c point to some GH receptor mRNA-containing neurons. In the ARC (a and b), *arrows* point to two of several neurons that appear to be double-labeled.

**TABLE 1.** Estimated percent of neurons double-labeled for NPY mRNA and GH receptor mRNA based on the signal to background grains ratio

Signal/Background	Percent of neurons double-labeled		
	Rat 1	Rat 2	Total
1	100%	100%	100%
2	96%	100%	98%
3	90%	93%	92%
4	76%	76%	76%
5	62%	61%	61%

**TABLE 2.** Estimated percent of neurons double-labeled for NPY mRNA and GH receptor mRNA based on the density of grains over the neurons

Grain density (Grains/1000 pixels)	Percent of neurons double-labeled	
	ARC	Cortex
2	100%	19%
4	98%	7%
6	96%	6%
8	92%	3%
10	85%	1%
16	61%	0%

Table 2 shows the results of the overall *signal density analysis* for both animals. In addition to the 330 neurons measured in the ARC of the two rats, an additional 163 were measured in the cortex and used in this analysis. Eighty-five percent of the NPY neurons in the ARC had a grain density of at least 10 grains/1000 pixels, whereas only 1% of the cortical cells reached this level. Frequency distribution histograms of signal density results for NPY mRNA-containing neurons in the ARC and the cortex are shown in Fig. 3.

#### Exp 2: GH regulation of NPY mRNA

Levels of GH in the serum of normal male rats were  $51 \pm 25$  ng/ml, whereas levels were less than 1 ng/ml in hypox rats. Levels of GH were within the physiological range ( $94 \pm 12$  ng/ml) in 5 of the hypox rats that were treated with rGH. In the sixth rGH-treated hypox rat, the minipump was severed at the time of sacrifice, contaminating the trunk blood that was collected.

As illustrated in Fig. 4, NPY mRNA levels varied as a function of GH state. The intensity of NPY mRNA labeling (as measured by gray level) was significantly less in hypox compared with intact animals ( $P < 0.05$ ). NPY mRNA signal levels were significantly greater in hypox animals treated with rGH compared with hypox animals treated with vehicle ( $P < 0.005$ ). NPY mRNA levels were not significantly different between the group of intact (vehicle-treated) animals and the group of hypox animals treated with rGH. Figure 5 shows low power, darkfield photomicrographs of emulsion-coated brain sections of the ARC that were labeled for NPY mRNA from representative intact/vehicle, hypox/vehicle and hypox/rGH animals.

#### Discussion

These results suggest that NPY neurons in the ARC are target cells for the direct action of GH and that part of this

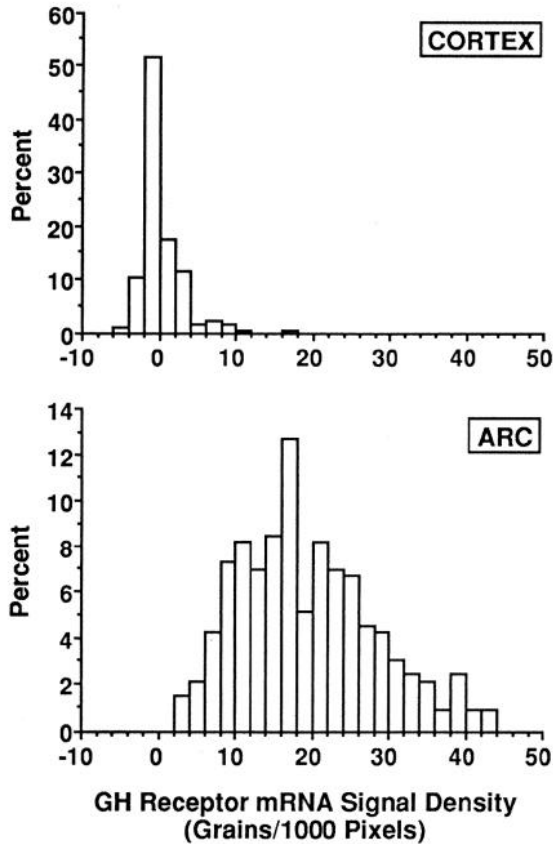


FIG. 3. Frequency distribution histograms of GH receptor signal density in NPY mRNA-containing cells in the ARC and the cortex.

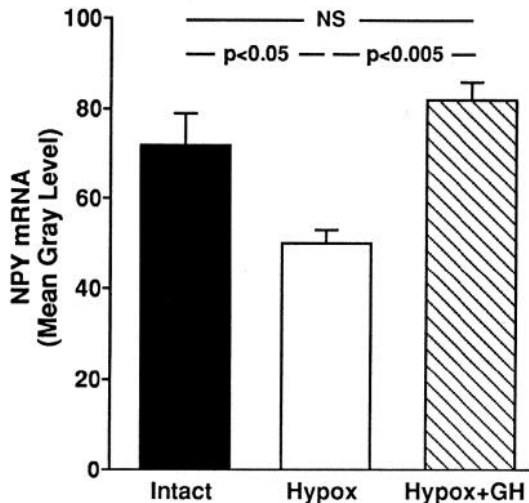


FIG. 4. Relative levels of NPY mRNA in the ARC of intact/vehicle, hypox/vehicle, and hypox/rGH animals. Values presented are the mean gray levels from a computerized image analysis system under the template shown in Fig. 1.

action involves the induction of NPY gene expression. First, we adduced evidence for the presence of GH receptor mRNA in the majority of ARC NPY neurons. Second, we report that NPY mRNA levels in the ARC were reduced after hypoph-

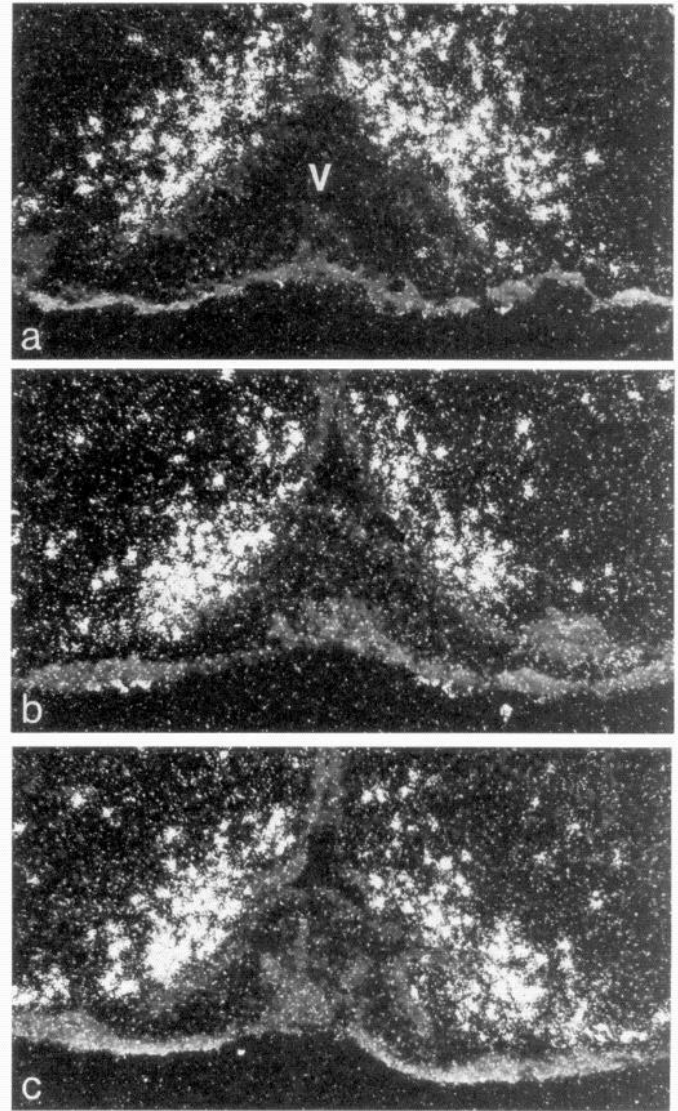


FIG. 5. Darkfield photomicrographs of NPY mRNA-containing neurons in the ARC of intact/vehicle (a), hypox/vehicle (b), and hypox/rGH (c) rats. The white dots are silver grains associated with concentrations of the radiolabeled NPY mRNA. V, Third ventricle.

ysectomy and that this reduction appeared to have been due to an absence of GH in these animals because it could be reversed by the administration of GH to the hypox animals. These results are consistent with the recent report by Kamagai and co-workers that GH induces the expression of *c-fos* mRNA in ARC NPY neurons (13). Furthermore, our results suggest that the induction of *c-fos* mRNA observed by Kamagai *et al.* is caused by a direct action of GH on NPY neurons and results in increased levels of NPY mRNA.

The inference that GH acts directly on NPY neurons is based on the colocalization of GH receptor mRNA and NPY mRNA in ARC neurons. Although visual inspection of double-labeled brain sections made it clear that most of the NPY mRNA-containing neurons in the ARC also contain GH receptor mRNA, the low level of GH receptor mRNA expression and relatively close packing of NPY mRNA-containing neurons made the quantitative analysis of coexpression es-

pecially challenging. To minimize the impact of overlapping grain clusters between NPY neurons, we restricted the grain counts associated with NPY neurons (signal grains) to only the area enclosed by the boundaries as indicated by the dark-blue digoxigenin reaction product in the cytoplasm. Because background levels appeared to be relatively high in the ARC, we wanted to make certain that background measurements did not underestimate the true background associated with NPY neurons in this region. Therefore, we counted background grains over the entire area surrounding the NPY neurons, even though this area was frequently punctuated by aggregations of grains that could not be attributed to identifiable NPY cell bodies. This approach would likely have provided a conservative estimate of the specific signal.

We used two different approaches to assess the frequency of GH receptor mRNA coexpression in NPY neurons. First, we compared the grain counts over each NPY neuron to the background level by calculating a *signal to background ratio*. Second, we compared the specific *grain density* (i.e. signal grain density minus the background grain density) between NPY cells in the ARC and those in the cortex. The basis for this comparison was the assumption that cortical NPY neurons do not express GH receptor mRNA. (This assumption cannot be proven, but we never observed any suggestion of double-labeling of cortical neurons with the cRNA probes for NPY mRNA and GH receptor mRNA.) We wanted to establish a threshold value to be used to differentiate between cells that unequivocally contain the GH receptor message (as indicated by the presence of many grains over their soma) and cells with grain counts that are too low to be certain that they express the GH receptor message. However, the numerical results derived from this process are dependent on the threshold that is selected, and the selection of any particular threshold is subjective and arbitrary. To illustrate this point, we have presented the analysis in a tabular form, which includes a range of threshold values (Tables 1 and 2). Although it is impossible to determine the precise percent of coexpression, these results suggest that most of the NPY neurons in the ARC express GH receptor mRNA, regardless of the threshold selected.

In this study we used hypophysectomy to determine the effect of GH on NPY mRNA in the ARC. Of course, removal of the pituitary also eliminates the secretion of other hypophysial hormones as well as those from target organs such as the thyroids, adrenals, and gonads. Indeed, Urban and co-workers (20) have shown that castration also reduces NPY mRNA in ARC neurons and that testosterone stimulates NPY mRNA in the ARC of castrated rats. Therefore, the reduction of testosterone secretion that occurred after removal of the pituitary would likely have contributed to the decrease in NPY mRNA we found in the hypox group. Nevertheless, the stimulation NPY mRNA in the ARC by GH that we observed in hypox animals clearly suggests a trophic role for GH as well.

Although it would appear likely that GH acts directly on NPY neurons in the ARC to regulate NPY gene expression, the physiological significance of the interaction between GH and NPY neurons remains to be elucidated. NPY neurons have been implicated in a number of important physiological

functions, including the regulation of reproduction, feeding behavior, and GH secretion (21–26). Within the hypothalamus, NPY neurons in the PeN, PVN, and DMN do not appear to express GH receptor mRNA. Thus, it seems likely that NPY neurons in the hypothalamus are heterogeneous, serving a variety of regulatory functions. GH has been shown to affect food intake (27), and NPY neurons in the ARC have been implicated in the regulation of feeding behavior (28); therefore, the idea that GH influences food intake through an effect on NPY neurons in the ARC makes some physiological sense. However, the close proximity of NPY neurons to the GHRH neurons suggests an interaction between these two groups of cells. If this were the case, then connections, either direct or indirect, should exist between NPY neurons and GHRH neurons in the ARC.

There is no direct evidence that NPY neurons make synaptic contact with GHRH neurons; however, electron microscopic immunohistochemical studies have demonstrated synaptic connections between NPY-containing axons and SS neurons in the PeN (29). This suggests that NPY neurons can influence the release of GH secretion by acting on PeN SS neurons. Whether these particular NPY-containing axons originate from cell bodies residing within the ARC remains to be established. If they do not, it would seem less likely that they are involved in mediating GH feedback but may perform other functions related to GH regulation. The fact that PeN SS neurons themselves express GH receptor mRNA would seem to obviate the need for NPY-mediated GH feedback on SS neurons; however, the interaction of NPY neurons with the feedback regulation of GH secretion may be more complex than we now realize. The feedback regulation of GHRH neurons in the ARC could be accomplished entirely through PeN SS neurons because there is anatomical evidence for connections between these two populations of neurosecretory cells (30, 31).

The observation that GH treatment increases intracellular levels of NPY mRNA in the ARC is consistent with the hypothesis that NPY neurons transduce at least part of the short-loop GH feedback signal to hypothalamic neurosecretory cells. Increased NPY mRNA expression is accompanied by increased *c-fos* mRNA levels (13), which suggests that these cells are induced by GH to synthesize and release NPY. To form part of a negative feedback loop regulating GH secretion, NPY would have to inhibit the release of GH, either by inhibiting GHRH secretion or by stimulating SS release. In several studies, the intracerebroventricular administration of NPY has been shown to inhibit circulating GH levels (21, 32), presumably due to an activation of SS neurons (22). A direct action of NPY on the pituitary is also conceivable; however, at the pituitary level, NPY is thought to stimulate GH secretion (33). Therefore, it is unlikely that NPY from neurons in the ARC acts at the pituitary to participate in the negative feedback regulation of GH.

The results presented here, when considered in the context of earlier observations that GH administration induces *c-fos* expression in ARC NPY neurons (13) and the NPY gene contains an AP-1 site (14), suggest a model to explain at least part of the effects of GH on NPY neurons in the ARC. According to this model, GH binds to GH receptors present in the membrane of NPY neurons in the ARC, inducing the

expression of *c-fos* mRNA and the production of Fos, the *c-fos* gene product. Fos in turn binds to the AP-1 site on the NPY gene and induces the expression of NPY mRNA. Increased *c-fos* and NPY mRNA expression suggests that GH induces NPY release from ARC NPY neurons. If this presumed increase in NPY release is involved in the short-loop feedback regulation of GH, the most likely target for the ARC NPY projections are SS neurons located in the PeN.

In summary, the majority of NPY neurons in the ARC express GH receptor mRNA, whereas NPY neurons at other sites within the hypothalamus do not. The binding of GH to its receptor induces the expression of NPY mRNA, presumably through a *c-fos*-dependent mechanism. Whether these NPY neurons play an important role in the regulation of GH secretion or participate in some other physiological system remains to be determined.

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### References

- Tannenbaum GS, Ling N 1984 The interrelationship of growth hormone (GH)-releasing factor and somatostatin in generation of the ultradian rhythm of GH secretion. *Endocrinology* 115:1952-1957
- Brazeau P, Ling N, Bohlen P, Esch F, Ying SY, Guillemin R 1982 Growth hormone releasing factor, somatocortin, releases pituitary growth hormone *in vitro*. *Proc Natl Acad Sci USA* 79:7909-7913
- Barinaga M, Yamonoto G, Rivier C, Vale W, Evans R, Rosenfeld M-G 1983 Transcriptional regulation of growth hormone gene expression by growth hormone-releasing factor. *Nature* 306:84-85
- Ibata Y, Okamura H, Makino S, Kawakami F, Morimoto N, Chihara K 1986 Light and electron microscopic immunocytochemistry of GRF-like immunoreactive neurons and terminals in the rat hypothalamic arcuate nucleus and median eminence. *Brain Res* 370:136-143
- Reichlin S 1983 Somatostatin. *N Engl J Med* 309:1495-1501; 1556-1563
- Kawano H, Daikoku S 1988 Somatostatin-containing neuron systems in the rat hypothalamus: retrograde tracing and immunohistochemical studies. *J Comp Neurol* 271:293-299
- Chihara K, Minamitani N, Kaji H, Arimura A, Fujita T 1981 Intraventricularly injected growth hormone stimulates somatostatin release into rat hypophysial portal blood. *Endocrinology* 109:2279-2281
- Katakami H, Downs TR, Frohman LA 1987 Effect of hypophysectomy on hypothalamic growth hormone-releasing factor content and release in the rat. *Endocrinology* 120:1079-1082
- Chomczynski P, Downs TR, Frohman LA 1988 Feedback regulation of growth hormone releasing hormone gene expression by growth hormone in rat hypothalamus. *Mol Endocrinol* 2:236-241
- Rogers KV, Vician L, Steiner RA, Clifton DK 1988 The effect of hypophysectomy and growth hormone administration on pre-somatostatin messenger ribonucleic acid in the periventricular nucleus of the rat hypothalamus. *Endocrinology* 122:586-591
- Burton KA, Kabigting EB, Clifton DK, Steiner RA 1992 Growth hormone receptor messenger ribonucleic acid distribution in the adult male rat brain and its colocalization in hypothalamic somatostatin neurons. *Endocrinology* 131:958-963
- Burton KA, Kabigting EB, Steiner RA, Clifton DK 1995 Identification of target cells for growth hormone's action in the arcuate nucleus. *Am J Physiol* 269:E716-E722
- Kamegai J, Minami S, Sugihara H, Higuchi H, Wakabayashi I 1994 Growth hormone induces expression of the *c-fos* gene on hypothalamic neuropeptide-Y and somatostatin neurons in hypophysectomized rats. *Endocrinology* 135:2765-2771
- Andersson G, Pahlman S, Parrow V, Johansson I, Hammerling U 1994 Activation of the human NPY gene during neuroblastoma cell differentiation: induced transcriptional activities of AP-1 and AP-2. *Cell Growth Differ* 5:27-36
- Paxinos G, Watson C 1986 *The Rat Brain in Stereotaxic Coordinates*, ed 2. Academic Press, Sydney
- Mathews LS, Enberg B, Norstedt G 1989 Regulation of rat growth hormone receptor gene expression. *J Biol Chem* 264:9905-9910
- Higuchi H, Yang HT, Sabol SL 1988 Rat neuropeptide Y precursor gene expression. *J Biol Chem* 263:6288-6295
- Chowen JA, Steiner RA, Clifton DK 1991 Semiquantitative analysis of cellular somatostatin mRNA levels by *in situ* hybridization histochemistry. *Methods Neurosci* 5:137-158
- Marks DL, Wiemann JN, Burton KA, Lent KL, Clifton DK, Steiner RA 1992 Simultaneous visualization of two cellular mRNA species by use of a new double *in situ* hybridization method. *Mol Cell Neurosci* 3:395-405
- Urban JH, Bauer-Dantoin AC, Levine JE 1993 Neuropeptide Y gene expression in the arcuate nucleus: sexual dimorphism and modulation by testosterone. *Endocrinology* 132:139-145
- McDonald JK, Lumpkin MD, Samson WK, McCann SM 1985 Neuropeptide Y affects secretion of luteinizing hormone and growth hormone in ovariectomized rats. *Proc Natl Acad Sci USA* 82:561-564
- Rettori V, Milenkovic L, Aguila MC, McCann SM 1990 Physiologically significant effect of neuropeptide Y to suppress growth hormone release by stimulating somatostatin discharge. *Endocrinology* 126:2296-2301
- Leibowitz SF 1991 Brain neuropeptide Y: an integrator of endocrine, metabolic and behavioral processes. *Brain Res Bull* 27:333-337
- Bray GA 1993 The nutrient balance hypothesis: peptides, sympathetic activity, and food intake. *Ann NY Acad Sci* 676:223-241
- Catzefflis C, Pierroz DD, Rohner-Jeanraud F, Rivier JE, Sizonenko PC, Aubert ML 1993 Neuropeptide Y administered chronically into the lateral ventricle profoundly inhibits both the gonadotropic and the somatotrophic axis in intact adult female rats. *Endocrinology* 132:224-234
- Kalra SP 1993 Mandatory neuropeptide-steroid signaling for the preovulatory luteinizing hormone-releasing hormone discharge. *Endocr Rev* 14:507-538
- Byatt JC, Staten NR, Salsgiver WJ, Kostelc JG, Collier RJ 1993 Stimulation of food intake and weight gain in mature female rats by bovine prolactin and bovine growth hormone. *Am J Physiol* 264:E986-992
- Akabayashi A, Wahlestedt C, Alexander JT, Leibowitz SF 1994 Specific inhibition of endogenous neuropeptide Y synthesis in arcuate nucleus by antisense oligonucleotides suppresses feeding behavior and insulin secretion. *Mol Brain Res* 21:55-61
- Hisano S, Tsuruo Y, Kagotani Y, Daikoku S, Chihara K 1990 Immunohistochemical evidence for synaptic connections between neuropeptide Y-containing axons and periventricular somatostatin neurons in the anterior hypothalamus in rats. *Brain Res* 520:170-177
- Daikoku S, Hisano S, Kawano H, Chikamori-Aoyama M, Kagotani Y, Zhang R, Chihara K 1988 Ultrastructural evidence of neuronal regulation of growth hormone secretion. *Neuroendocrinology* 47:405-415
- Horvath S, Palkovits M, Gorcs T, Arimura A 1989 Electron microscopic immunocytochemical evidence for the existence of bidirectional synaptic connections between growth hormone-releasing hormone- and somatostatin-containing neurons in the hypothalamus of the rat. *Brain Res* 481:8-15
- Harfstrand A, Eneroth P, Agnati L, Fuxe K 1987 Further studies on the effects of central administration of neuropeptide Y on neuroendocrine function in the male rat: relationship to hypothalamic catecholamines. *Regul Pept* 17:167-179
- Chabot JG, Enjalbert A, Pelletier G, Dubois PM, Morel G 1988 Evidence for a direct action of neuropeptide Y in the rat pituitary gland. *Neuroendocrinology* 47:511-517