

# Leptin Receptor mRNA Identifies a Subpopulation of Neuropeptide Y Neurons Activated by Fasting in Rat Hypothalamus

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The decline of leptin (Ob protein) concentrations during fasting is implicated as a signal for increasing the expression of the orexigenic peptide neuropeptide Y (NPY) in the hypothalamus. To test the hypothesis that the effects of food intake on arcuate nucleus NPY activation are mediated by leptin, we performed simultaneous triple in situ hybridization colocalization studies to determine whether the subset of NPY neurons that are activated by fasting preferentially expresses the long form of the leptin receptor (Ob-Rb). Thus, mRNAs encoding NPY and pro-opiomelanocortin (POMC) were colocalized in the arcuate nucleus of fed and fasted rats by fluorescence in situ hybridization in combination with isotopic in situ hybridization for Ob-Rb mRNA. In fed animals, 47% of arcuate nucleus neurons containing NPY mRNA also contained Ob-Rb mRNA, compared with 79% of POMC neurons ( $P < 0.01$ ). After a 2-day fast, the number of arcuate nucleus neurons with NPY mRNA increased 50% ( $P < 0.05$ ); the number of these that coexpressed Ob-Rb increased twofold ( $P = 0.013$ ). Furthermore, Ob-Rb mRNA hybridization in individual NPY neurons increased by 64% ( $P < 0.02$ ). In contrast, the number of POMC neurons that coexpressed Ob-Rb was unchanged. A significant interpretation of these findings is that the NPY neurons that do not express detectable levels of Ob-Rb mRNA are not activated by fasting, whereas the NPY neurons that are activated by fasting are the ones that express Ob-Rb. These data demonstrate a significant physiological difference between NPY neurons that express Ob-Rb and those that do not. The results support the conclusion that the effect of food intake on NPY neurons is mediated by the direct action of leptin via Ob-Rb receptors expressed by these NPY cells. The results also indicate that expression of Ob-Rb is a defining phenotypic characteristic of the subset of arcuate nucleus NPY neurons that are activated by fasting and play a central role in the adaptive response to negative energy balance. *Diabetes* 48:828–833, 1999

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Received for publication 19 August 1998 and accepted in revised form 23 December 1998.

AGRP, agouti-related protein; FISH, fluorescence in situ hybridization; NPY, neuropeptide Y; POMC, pro-opiomelanocortin.

The regulation of adiposity by the central nervous system involves changes in the expression of hypothalamic neuropeptides in response to changes in energy balance (1,2). Neuropeptide Y (NPY) is of special interest because it acts as an anabolic effector, potentially stimulating food intake when released into the hypothalamus in the vicinity of the paraventricular nucleus (3,4). When animals are in a state of negative energy balance, such as during a fast, NPY biosynthesis in the arcuate nucleus (5,6) as well as NPY transport to the paraventricular nucleus and its release there (7) are both markedly increased, thereby facilitating increased food intake. Although NPY is expressed in many brain regions, activation of NPY gene expression in response to fasting is a unique feature of NPY neurons in the hypothalamic arcuate nucleus (8). In addition, arcuate nucleus NPY neurons also express agouti-related protein (AGRP) (9), an endogenous melanocortin receptor antagonist that stimulates food intake by decreasing melanocortin action (10), and the parallel expression of both NPY and AGRP in these neurons is simultaneously activated by fasting (9). These arcuate nucleus NPY/AGRP neurons, therefore, represent a unique NPY neuronal subtype that is activated by fasting to stimulate food intake (9). The present study addressed a putative mechanism that could directly link the effects of fasting to the activation of these NPY neurons.

We hypothesized that leptin (Ob protein) deficiency in the hypothalamus (as a consequence of decreased plasma leptin levels) plays a key role in the activation of arcuate nucleus NPY neurons during a period of food restriction. NPY gene expression in the arcuate nucleus is increased in response to low leptin levels, as occurs in genetic leptin deficiency (*ob/ob* mice) (11,12) and fasting (13). Conversely, administration of leptin to leptin-deficient or fasted rodents inhibits this increase of hypothalamic NPY expression and reduces food intake (13–19). Furthermore, mRNA encoding the major signaling form of the leptin receptor (Ob-Rb) (20) as well as the Ob-Rb receptor protein have been identified in NPY neurons in the arcuate nucleus (21–24). However, whether the particular arcuate nucleus NPY neurons that express Ob-Rb mRNA are the same ones that are activated by fasting is not known. Furthermore, it is not known whether the effect of fasting on NPY neurons is direct or is mediated by leptin action on other cells. These represent major gaps in our understanding of the mechanism that links changes in food intake to arcuate nucleus NPY gene expression. The hypoth-

esis that the effects of fasting on arcuate nucleus NPY gene expression are due to the direct action of leptin on these cells would be supported by finding evidence for expression of Ob-Rb in arcuate nucleus NPY neurons that are activated during a fast.

To address the question of whether the effects of fasting on arcuate nucleus NPY expression are mediated by the direct action of leptin, we used a triple in situ hybridization procedure that simultaneously colocalized mRNAs for NPY and proopiomelanocortin (POMC) by fluorescence in situ hybridization (FISH) and Ob-Rb mRNA by isotopic in situ hybridization, in the brain of fed and fasted rats. In situ hybridization for POMC mRNA was included as a control because Ob-Rb mRNA has been colocalized with POMC mRNA in the arcuate nucleus (25), but fasting lowers, whereas leptin increases, POMC mRNA levels (26–28). Because arcuate nucleus POMC gene expression is regulated differently than that of NPY, POMC neurons were not expected to be activated during fasting. The results suggest that among the population of NPY neuron cell bodies in the arcuate nucleus, Ob-Rb is expressed by the subset that was activated to increase NPY gene expression during fasting.

## RESEARCH DESIGN AND METHODS

**Experimental conditions.** Male Wistar rats (280–300 g) (Simonsen Labs, Gilroy, CA) were housed individually and maintained on a 12/12-h day/night cycle (7:00 A.M.–7:00 P.M.) with ad lib access to standard rodent food and water before the study. For the experiment, rats were fasted by removing their food beginning at 0900 in the morning and continuing for 48 h, but they were allowed free access to water ( $n = 4$ ). Control animals were given free access to food and water ( $n = 4$ ). At the end of the fast, animals were euthanized in the morning by decapitation under CO<sub>2</sub> inhalation. All procedures were approved by the animal research committees of the Veterans Affairs Puget Sound Health Care System and the University of Washington.

**Tissue preparation.** Brains were removed immediately after decapitation between 0800–1200, frozen on dry ice, sectioned in a coronal plane at 14  $\mu$ m with a cryostat, mounted on RNase-free slides, and treated with 4% paraformaldehyde, acetic anhydride, ethanol, and chloroform (13). For each animal, six slides (12 brain sections) containing hypothalamus in the region 2.0–3.5 mm posterior to bregma (29) were selected for hybridization. Care was taken to ensure that the sections were anatomically matched among animals. All brain slices were concurrently prepared for hybridization and used in the same assay.

**Riboprobe preparation.** Riboprobes for peptide mRNAs were transcribed from cDNA templates for NPY (30) and POMC (27,31). The Ob-Rb template is based on nucleotides 2899–3251 encoding the cytoplasmic tail of the Ob-Rb (32) and is specific for Ob-Rb mRNA in rat. Antisense riboprobes were transcribed using appropriate RNA polymerases and either a digoxigenin (for POMC) or biotin (for NPY) labeling mix (Boehringer Mannheim, Indianapolis, IN). The Ob-Rb riboprobe was transcribed using <sup>33</sup>P-UTP (Amersham, Arlington Heights, IL) as described previously (24). Unincorporated label was separated using a QIAquick Nucleotide Removal Kit (Qiagen, Santa Clarita, CA). Riboprobe yield was determined using optical density at 260 nm for the nonradioactive probes and from radioactivity counting for the Ob-Rb mRNA probe. Melting temperature calculations assumed that the transcription reaction produced full-length transcripts.

**In situ hybridization.** The study used a triple in situ hybridization procedure in which mRNAs for NPY and POMC were detected by FISH, and Ob-Rb mRNA was detected by isotopic in situ hybridization. The in situ hybridization procedures and controls followed a standard protocol (24), except that the three probes were applied simultaneously in a mixture and then subjected to different detection protocols. To detect POMC mRNA hybridization, slides were immersed in 0.1 mol/l Tris/150 mmol/l NaCl (pH 7.4) and then in mouse anti-digoxigenin monoclonal IgG (Jackson ImmunoResearch, West Grove, PA) (1:5,000) in Tris/NaCl buffer containing 0.05% Triton X-100 and 1% normal goat serum for 3 h at 37°C. After a 3× wash in Tris/NaCl/Triton X-100 buffer for 5 min, slides were immersed in goat anti-mouse IgG conjugated with Cy3 (Jackson ImmunoResearch) (1:200) in Tris/NaCl buffer with 0.05% Triton X-100. The bright fluorescence produced by the Cy3 fluorochrome was adequate for detecting digoxigenin-labeled riboprobes (9). After a 1-h incubation at 37°C, slides were again washed in buffer. Visualization of the biotin-labeled NPY riboprobe was accomplished with a TSA (tyramide signal amplification) Indirect Kit (New England Nuclear, Boston, MA), using the manufacturer's protocol. The TSA method was used to increase the Cy2 fluorescence

signal resulting from hybrids with the biotinylated NPY riboprobe (9). Controls included substitution of normal serum for unlabeled second antibodies and primary antibodies. Slides were incubated with streptavidin-horseradish peroxidase followed by biotinyl tyramide and streptavidin-Cy2 conjugate (Molecular Probes, Eugene, OR). After representative slides were examined to confirm the presence of FISH, the slides were coated with NTB2 nuclear track emulsion, exposed for 14 days, developed, and then coverslipped with a glycerol mounting medium containing 1  $\mu$ g/ml Hoechst 33342 nuclear stain (Sigma, St. Louis, MO) that permitted identification of cells and anatomical landmarks. FISH was observed with a Zeiss Axioplan fluorescence microscope, using a fluorescein isothiocyanate filter set for Cy2 and a rhodamine filter set for Cy3. Extensive autoradiographic controls showed no evidence of chemographic artifacts produced by any of the reagents or procedures.

**Quantitation.** Quantitative analysis was done with an MCID M2 image analysis system (Imaging Research, St. Catharines, Ontario), using a Hamamatsu C4880 CCD camera. Images were acquired at 12-bit gray level resolution and displayed in pseudocolor for analysis, with NPY represented by green (Cy2 fluorescence) and POMC by red (Cy3 fluorescence). Autoradiographic grains were displayed and imaged in dark-field optics. The MCID image analysis program was used to count numbers of FISH<sup>+</sup> NPY and POMC neurons. Sampling was done bilaterally in the arcuate nucleus with a 40× objective. Results were standardized to the number of labeled or unlabeled cells in a microscopic field of the arcuate nucleus at 400× magnification. To measure colocalization of mRNAs for Ob-Rb and NPY, the FISH image for NPY mRNA was first digitized into an image channel, while the dark-field image of grains representing Ob-Rb hybridization in the same sample field was digitized into a separate image channel (images in the separate channels remained in register). The MCID image analysis program measured colocalization by first highlighting the labeled fluorescent cells in the NPY image channel and then counting grains corresponding to these same cells from the dark-field image in the second channel, and it expressed the results as grains per cell. Colocalization of POMC and Ob-Rb mRNAs followed the same procedure. Nonspecific grain density was obtained in the cerebral cortex. FISH-labeled NPY and POMC neurons in the arcuate nucleus were considered to be Ob-Rb mRNA<sup>+</sup> if the number of grains per cell body exceeded background levels. For statistical analysis, pairwise comparisons of means were done with the two-tailed unpaired Student's *t* test. The null hypothesis of equal means was rejected at the  $P = 0.05$  level of significance. Data are expressed as means  $\pm$  SE.

## RESULTS

Neuronal cell bodies containing mRNAs for NPY and POMC were readily identified by bright fluorescence in the arcuate nucleus after in situ hybridization with riboprobes and fluorescence detection (FISH) (Fig. 1). As expected, most of the neurons with NPY hybridization signal were clustered adjacent to the third ventricle, whereas most of the neurons containing POMC mRNA signal were located in the lateral region of the arcuate nucleus. Furthermore, FISH signal for NPY and POMC mRNA was not observed within the same cells. All in situ hybridization signals were abolished by RNase treatment. Likewise, in situ hybridization signals for POMC mRNA were absent when normal mouse serum was substituted for the anti-digoxigenin antiserum, and the NPY mRNA hybridization signal was abolished by omitting streptavidin-horseradish peroxidase in the tyramide amplification protocol. These controls demonstrated the specificity of the FISH method and the absence of nonspecific effects.

To verify that the hypothalamus of the fasted rats responded appropriately to negative energy balance by increasing NPY expression, the number of neurons that contained FISH signal for NPY was counted in the arcuate nucleus of fed and fasted rats (Fig. 2). Quantitative measurements showed that the number of NPY mRNA<sup>+</sup> cell bodies in the arcuate nucleus increased by 50% during fasting ( $33.7 \pm 2.4$  vs.  $22.4 \pm 1.6$  NPY mRNA<sup>+</sup> cells/field;  $P < 0.05$ ). In contrast, the number of labeled POMC mRNA<sup>+</sup> cells detected by FISH was unchanged by fasting ( $25.2 \pm 1.7$  vs.  $23.1 \pm 2.3$  POMC mRNA<sup>+</sup> cells/field).

To determine whether arcuate nucleus neurons that increased NPY transcription during fasting also expressed the

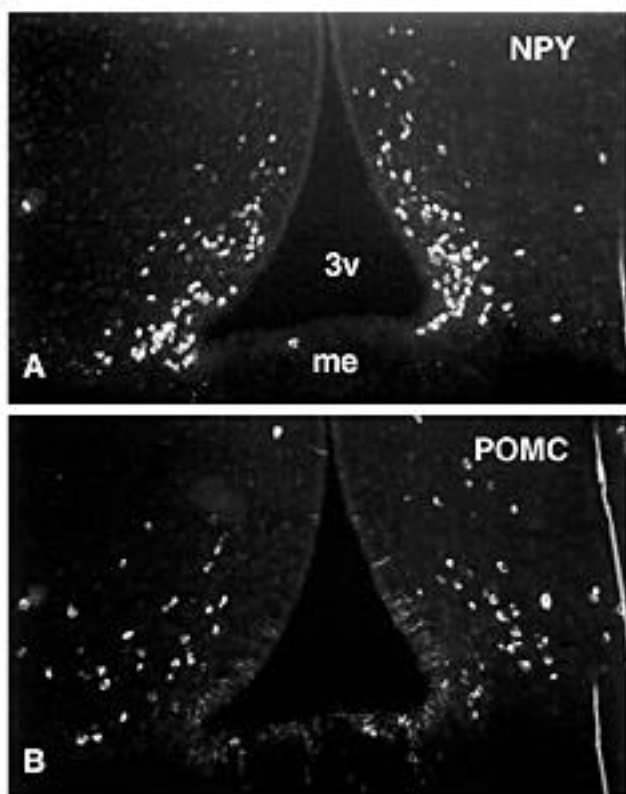


FIG. 1. Single section of rat arcuate nucleus showing simultaneous FISH localization of NPY mRNA (A) and POMC mRNA (B) in a fasted rat. 3v, third ventricle; me, median eminence.

leptin receptor gene, we counted separately the number of neurons with positive in situ hybridization for mRNAs encoding NPY and Ob-Rb, as well as the number that showed in situ hybridization for both NPY and Ob-Rb mRNA. Colocalization of POMC and Ob-Rb mRNAs was measured similarly. High concentrations of autoradiographic grains (in comparison to background levels) representing Ob-Rb mRNA were seen over many NPY (Fig. 3) and POMC cell bodies in the arcuate nucleus. Among the fed animals, only 47% of NPY neurons coexpressed Ob-Rb mRNA compared with 79% for POMC neurons ( $P < 0.01$ ). Quantitative measurements of Ob-Rb mRNA<sup>+</sup> cells in the arcuate nucleus revealed that the number of NPY neurons that coexpressed Ob-Rb mRNA was increased twofold in fasted rats as compared with fed rats ( $21.3 \pm 2.9$  vs.  $10.3 \pm 1.6$  cells/field;  $P = 0.013$ ), whereas the number of arcuate nucleus NPY neurons without Ob-Rb mRNA was unchanged by fasting ( $12.4 \pm 2.1$  vs.  $12.1 \pm 1.6$  cells/field) (Fig. 4). In contrast, both the total number of POMC neurons as well as the percentage of POMC neurons that coexpressed Ob-Rb mRNA were not altered by fasting (Fig. 4) ( $21.3 \pm 1.8$  vs.  $18.5 \pm 3.0$  cells/field). Furthermore, NPY neurons in the hippocampus, cerebral cortex, and thalamus did not show expression of Ob-Rb above background levels in fed or fasted animals (Fig. 5).

Because the autoradiographic grain density per cell can be considered to be proportional to mRNA expression, the numbers of grains were counted for the NPY and POMC cells that coexpressed Ob-Rb mRNA to determine whether Ob-Rb mRNA levels in NPY or POMC neurons were altered by fasting. The results showed that Ob-Rb mRNA hybridization in

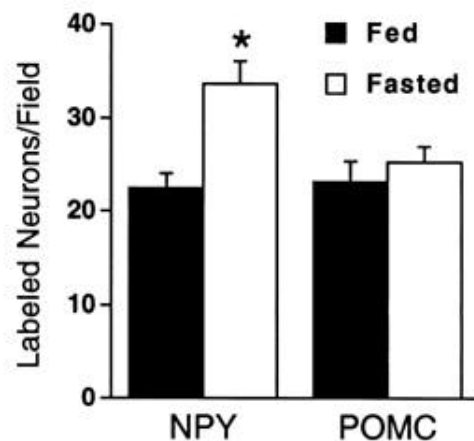


FIG. 2. The number of arcuate nucleus neurons expressing NPY mRNA, as detected by in situ hybridization, was elevated in fasted rats, whereas there was no change in the number of neurons expressing POMC mRNA. \* $P < 0.05$ .

individual arcuate nucleus NPY neurons was increased by 64% during fasting ( $P < 0.02$ ) (Fig. 6). In contrast, the mean data for the POMC neurons showed a slight decline (9%) in Ob-Rb mRNA per cell, although the difference was not significant. Interestingly, levels of Ob-Rb mRNA were higher by 2-fold (fasted rats) to 3.5-fold (fed rats) in the POMC neurons in comparison to the NPY neurons ( $P < 0.01$ ). Thus, although the POMC neurons appeared to express more leptin receptor mRNA than did the NPY neurons, fasting was associated with increased Ob-Rb mRNA levels only in NPY cells.

## DISCUSSION

Although FISH enabled us to count the number of cells that contained NPY and POMC mRNA, the amount of hybridized mRNA per cell could not be measured (unlike the autoradiographic images produced by in situ hybridization with radioactive in situ hybridization). Consequently, we could not determine quantitatively the effect of fasting on the amounts of NPY mRNA and POMC mRNA per cell. The FISH method nevertheless provided a unique and rigorous approach for simultaneous measurement of the numbers of NPY and POMC neurons in the arcuate nucleus, and when combined with isotopic in situ hybridization for Ob-Rb mRNA, the FISH method enabled us to measure the numbers of NPY and POMC neurons that coexpressed Ob-Rb in fed and fasted states.

We hypothesized that the mechanism underlying the changes in arcuate nucleus NPY gene expression in response to changes in food intake involves the direct action of leptin on arcuate nucleus NPY neurons. According to this model, the arcuate nucleus NPY neurons that increase NPY mRNA transcription during fasting should also express Ob-Rb. In contrast, NPY neurons in the arcuate nucleus (and elsewhere) that do not respond to fasting should lack Ob-Rb expression. Using rats fasted for 48 h, we found a significant twofold increase in the number of neurons that coexpressed NPY mRNA and Ob-Rb mRNA, whereas the number of arcuate nucleus NPY neurons lacking Ob-Rb mRNA did not change during fasting. These results are consistent with both of the above predictions and indicate that expression of Ob-Rb may be a phenotypic characteristic of a subset of arcuate nucleus NPY neurons that are activated by fasting.

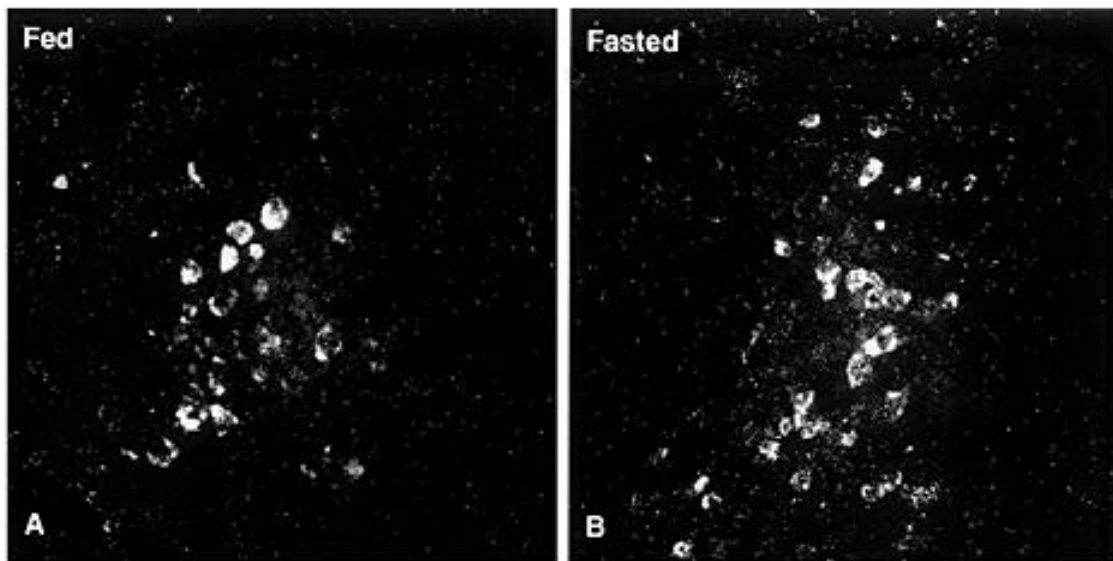


FIG. 3. Representative images of arcuate nucleus of fed (*A*) and fasted (*B*) rats, showing NPY neuronal cell bodies (detected by FISH) and Ob-Rb mRNA in situ hybridization signal (detected by autoradiographic grains). Dense concentrations of grains over NPY cell bodies are not obvious here because of the bright FISH signal of NPY cell bodies; however, this did not affect the counting of grains during analysis because the grains and cells were imaged and counted in separate image channels.

One way that the number of NPY mRNA<sup>+</sup> cells with Ob-Rb mRNA could have increased while the number without Ob-Rb remained constant would be if all of the “new” NPY cells (i.e., those that were activated to express more NPY mRNA during fasting) also expressed Ob-Rb mRNA during the fed state, when many of them were not detectable by NPY in situ hybridization. In support of this interpretation, we observed many arcuate nucleus neurons that were Ob-Rb mRNA<sup>+</sup> but NPY mRNA<sup>-</sup> (as well as POMC mRNA<sup>-</sup>). Presumably, some of the NPY<sup>-</sup> Ob-Rb<sup>+</sup> cells in the fed animals were activated to increased NPY gene transcription during fasting, whereby they would have been counted among the NPY<sup>+</sup> Ob-Rb<sup>+</sup> cells. Alternatively, the same result could have been obtained if some of the activated NPY cells in the fasted animals did not have Ob-Rb and if the number of such cells was precisely offset by an increase in the number of NPY neurons with Ob-Rb mRNA that did not express Ob-Rb mRNA in the fed state but did so during fasting. Because the latter explanation seems unlikely, we prefer to conclude that the neurons that showed higher NPY transcription during fasting are the same subset that expressed Ob-Rb mRNA. This interpretation suggests that the arcuate nucleus also has a subpopulation of NPY neurons that are not activated by fasting. We infer that the latter NPY neurons are the ones that did not express Ob-Rb mRNA. These latter neurons are presumably involved in functions that are not sensitive to leptin.

An alternative interpretation is imposed by the limitation of the FISH method to determine only whether the NPY mRNA concentration in a cell is above or below the limit of detection. It might be expected that the Ob-Rb<sup>+</sup> cells would in fact express less NPY mRNA than Ob-Rb<sup>-</sup> cells, even in the fed state, since leptin inhibits NPY gene expression (8). However, we did not measure NPY mRNA levels in individual neurons in this study. Thus, it is conceivable that NPY mRNA levels in Ob-Rb<sup>+</sup> neurons were present in concentrations lower (and below the limit of detection) than those in Ob-Rb<sup>-</sup> neurons. If in this case fasting did induce an increase

in NPY mRNA in the Ob-Rb<sup>-</sup> neurons, then the NPY mRNA levels in the latter neurons may have been above the threshold of detectability (even in the fed state). If so, this “activation” would have been overlooked because no increase in the number of Ob-Rb<sup>-</sup> NPY<sup>+</sup> cells would have been detectable. This explanation seems unlikely, however, in light of our hypothesis, because the Ob-Rb<sup>-</sup> neurons would not be expected to be leptin sensitive (i.e., they should not change NPY expression during fasting).

Finding a greater number of arcuate nucleus neurons with detectable levels of NPY mRNA in fasted rats compared with fed animals suggests that many arcuate nucleus NPY neurons have insufficient NPY transcriptional activity to be detected by in situ hybridization during ad libitum feeding. During fasting, however, these neurons are activated to increase

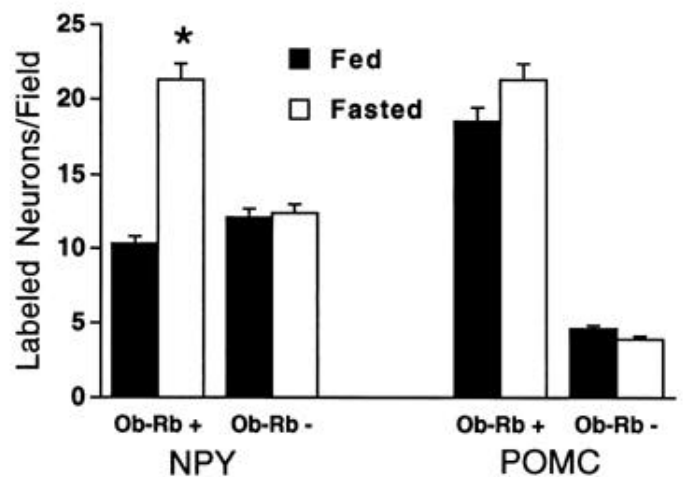


FIG. 4. Neuron cell bodies per sampled fields in arcuate nucleus of fed and fasted rats, showing numbers of neurons detected with NPY and POMC mRNAs (FISH) that also contained mRNA for Ob-Rb (Ob-Rb<sup>+</sup>) in comparison to those that were lacking Ob-Rb mRNA (Ob-Rb<sup>-</sup>) (isotopic in situ hybridization). \**P* = 0.013.

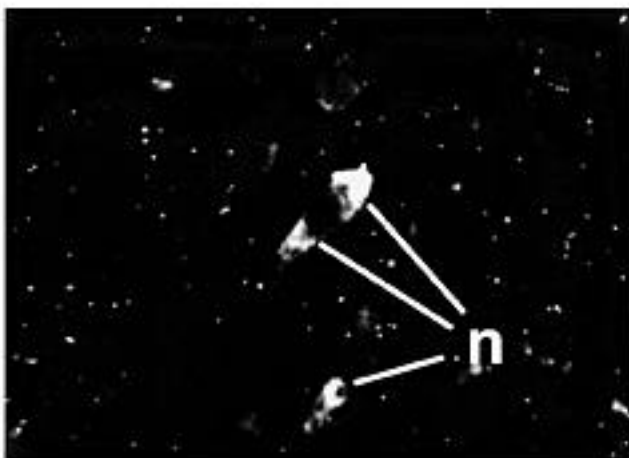


FIG. 5. Representative neurons (n) with NPY mRNA (detected by FISH) in somatosensory cerebral cortex from a fasted rat, showing both lack of detectable in situ hybridization signal for Ob-Rb (autoradiographic grains) in neurons and low background grain density.

NPY gene transcription, and thus NPY mRNA can be detected by in situ hybridization in more of these cells than in cells in the fed state. This interpretation provides a plausible explanation for our recent finding that fasting increases the number of neurons that actively transcribe the NPY gene in the arcuate nucleus of mice containing a *lacZ* reporter gene fused to the NPY promoter (8).

Therefore, the results support the hypothesis that leptin tonically inhibits NPY gene expression in a subset of arcuate nucleus NPY neurons, and that the reduction in leptin levels during fasting (33,34) activates these neurons. This hypothesis is also consistent with the finding that arcuate nucleus NPY mRNA is overexpressed in leptin-deficient *ob/ob* mice (35) and in leptin-resistant *db/db* mice (12) and *fa/fa* rats (36). This interpretation does not preclude the potential contributions of factors other than leptin (i.e., insulin, glucocorticoids) on these NPY cells. Nor does it rule out the possibility that non-leptin-sensitive NPY neurons in the arcuate nucleus may participate in the hypothalamic response to altered energy balance, or that other leptin-sensitive neuronal systems in the hypothalamus (13,25–28,37–42) may facilitate the response of NPY neurons to low leptin levels.

The number of neurons with POMC mRNA (as detected by FISH) did not change during fasting, although POMC mRNA in the arcuate nucleus (measured by radioactive in situ hybridization) decreases in this setting (27,28), and leptin administration elevates arcuate nucleus POMC mRNA levels (26–28). Because almost 80% of the arcuate nucleus POMC neurons in this study also expressed Ob-Rb mRNA, it is likely that leptin acts directly on most arcuate nucleus POMC neurons. However, the reduction of POMC mRNA levels in individual neurons during fasting appears to have been insufficient to reduce the number of POMC neurons that were detected by FISH. An interesting finding is that Ob-Rb mRNA levels changed during fasting in NPY cells but not in POMC cells. This suggests that the two cell types may have different mechanisms for regulating Ob-Rb gene expression. Moreover, this finding suggests that the increase of Ob-Rb mRNA levels in the arcuate nucleus during fasting (24) occurs preferentially in NPY neurons.

A significant finding of this study is that NPY neurons that do not express Ob-Rb (or express it below detectable levels)

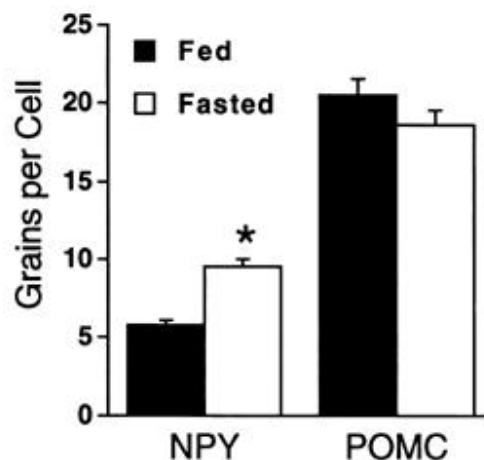


FIG. 6. Increased in situ hybridization signal (grains per cell) for Ob-Rb mRNA during fasting in neuron cell bodies containing in situ hybridization signal for NPY mRNA but not for POMC mRNA in arcuate nucleus. \* $P < 0.01$ .

are not activated by fasting. Thus, the data convincingly demonstrate a difference between NPY neurons that are Ob-Rb<sup>+</sup> and those that are Ob-Rb<sup>-</sup>. The basis for this difference could be either 1) that Ob-Rb<sup>-</sup> NPY neurons do not respond to fasting or 2) that Ob-Rb<sup>-</sup> NPY neurons in the fed state express higher levels of NPY so that any increase in NPY mRNA in these cells during fasting would not be detected by the FISH method. If the latter case is true, however, it would seem unlikely that this putative effect of fasting would be mediated by direct effects of leptin, since these cells appear to lack Ob-Rb (although it is conceivable that the effect could be mediated by another leptin receptor splice variant). The conclusion that the Ob-Rb<sup>+</sup> NPY neurons are the ones that respond to fasting would be supported by quantitative data showing that levels of NPY mRNA are similar in Ob-Rb<sup>+</sup> and Ob-Rb<sup>-</sup> neurons in the fed state but that NPY mRNA levels are elevated only in the Ob-Rb<sup>+</sup> neurons during fasting. This determination was not possible with the FISH method used in the present study.

Nevertheless, the finding that Ob-Rb<sup>+</sup> and Ob-Rb<sup>-</sup> NPY neurons in the arcuate nucleus are different in their response to fasting represents an important advance in our understanding of the hypothalamic role of leptin in regulation of food intake. The results support the conclusion that the effect of food intake on NPY neurons is mediated by the direct action of leptin via Ob-Rb receptors expressed in these cells. The results are also consistent with the hypothesis that expression of Ob-Rb mRNA is a defining phenotypic characteristic of this subset of neurons that increase expression of NPY and AGRP in response to fasting (9). An important inference from these results is that if the effects of fasting on NPY neurons occur only in NPY neurons that express Ob-Rb, then either all of the effects of fasting on NPY expression may be mediated by leptin or at least the influence of other factors (such as insulin) probably act through leptin-sensitive neurons.

#### ACKNOWLEDGMENTS

This research was supported by the Department of Veterans Affairs Merit Review and Career Scientist Programs and by National Institutes of Health Grants DK-17047, DK-52989, and NS32273.

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