

## Identification of Targets of Leptin Action in Rat Hypothalamus

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### Abstract

The hypothesis that leptin (OB protein) acts in the hypothalamus to reduce food intake and body weight is based primarily on evidence from leptin-deficient, *ob/ob* mice. To investigate whether leptin exerts similar effects in normal animals, we administered leptin intracerebroventricularly (icv) to Long-Evans rats. Leptin administration (3.5  $\mu$ g icv) at the onset of nocturnal feeding reduced food intake by 50% at 1 h and by 42% at 4 h, as compared with vehicle-treated controls (both  $P < 0.05$ ). To investigate the basis for this effect, we used in situ hybridization (ISH) to determine whether leptin alters expression of hypothalamic neuropeptides involved in energy homeostasis. Two injections of leptin (3.5  $\mu$ g icv) during a 40 h fast significantly decreased levels of mRNA for neuropeptide Y (NPY, which stimulates food intake) in the arcuate nucleus ( $-24\%$ ) and increased levels of mRNA for corticotrophin releasing hormone (CRH, an inhibitor of food intake) in the paraventricular nucleus (by 38%) (both  $P < 0.05$  vs. vehicle-treated controls). To investigate the anatomic basis for these effects, we measured leptin receptor gene expression in rat brain by ISH using a probe complementary to mRNA for all leptin receptor splice variants. Leptin receptor mRNA was densely concentrated in the arcuate nucleus, with lower levels present in the ventromedial and dorsomedial hypothalamic nuclei and other brain areas involved in energy balance. These findings suggest that leptin action in rat hypothalamus involves altered expression of key neuropeptide genes, and implicate leptin in the hypothalamic response to fasting. (*J. Clin. Invest.* 1996. 98:1101–1106.) Key words: obesity • body adiposity • central nervous system • *ob* gene

### Introduction

The hormonal product of the *ob* gene, leptin, is proposed to participate in the negative feedback control of body adiposity (1). Leptin deficiency due to mutation of the *ob* gene (in *ob/ob*

mice) (1) and leptin resistance due to mutation of the leptin receptor (in *db/db* mice [2] and *fa/fa* rats [3]) both cause overeating and severe obesity. Furthermore, systemic leptin administration reduces food intake and body weight in *ob/ob* mice (4–7) and in normal mice. Combined with the observation that plasma leptin levels vary in proportion to adiposity in normal and obese animals and humans (8–10), these data implicate leptin in normal body weight regulation. To determine if the brain of normal animals is sensitive to leptin, we hypothesized that its anorexic effects should be enhanced after administration directly into the cerebral ventricles. Therefore, we first sought to determine if intracerebroventricular (icv) leptin administration inhibits food intake in Long-Evans rats at doses that are ineffective following peripheral administration. Second, we wished to test the hypothesis that leptin's effects on food intake and energy balance in normal animals involves altered hypothalamic neuropeptide gene expression. Since neuropeptide Y (NPY) is overexpressed in the hypothalamus of *ob/ob* (11), as well as *db/db* mice (12) and in the *fa/fa* rat (13), impaired leptin signaling may stimulate NPY gene expression. This hypothesis is supported by the observation that leptin treatment lowers NPY mRNA levels in the arcuate nucleus of *ob/ob* mice (14, 15).

We therefore hypothesized that in normal animals, leptin inhibition of hypothalamic NPY gene expression contributes to its weight-reducing action. Since fasting stimulates NPY gene expression in the arcuate nucleus of normal rats (16, 17), we reasoned that leptin inhibition of NPY may be detected more readily in fasted, as compared with fed animals. Moreover, many of leptin's effects on energy balance (e.g., reduced food intake and increased metabolic rate) (5, 6) are opposite to those that occur in fasted animals (e.g., stimulation of food intake and reduced energy expenditure) (18). Thus, we proposed that leptin may inhibit responses that are normally engaged during fasting, and that the effect of fasting to lower the circulating leptin level (8) is required for these adaptive responses to occur. In addition to its effects to stimulate hypothalamic NPY production, fasting reduces expression of corticotrophin releasing hormone (CRH) in the PVN (19, 20). Since CRH acts in this brain area to inhibit food intake and to increase energy expenditure (21, 22), whereas NPY exerts the opposite effects (23, 24), these changes in hypothalamic neuropeptide gene expression induced by fasting are implicated in the associated feeding and autonomic responses. We hypothesized, therefore, that leptin administration directly into the brain of fasted rats should increase hypothalamic expression of

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1. Abbreviations used in this paper: CRH, corticotrophin releasing hormone; icv, intracerebroventricularly; ISH, in situ hybridization; NPY, neuropeptide Y; PVN, paraventricular nucleus.

CRH and decrease expression of NPY, leading to an attenuated feeding response. Further, we hypothesized that if leptin acts directly on hypothalamic neurons to induce changes in neuropeptide gene expression, leptin receptor mRNA should be detectable in these key brain areas.

## Methods

### Study animals and procedures

Male Long-Evans rats (350–450 grams) 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. Obese Zucker rats were obtained from Hoffmann-LaRoche and housed in rectangular clear plastic cages. Unless otherwise specified, animals were given free access to food (pelleted rat chow) and water at all times. All procedures were performed in accordance with institutional guidelines for animal care at the University of Washington.

**Cannula placement.** Rats were anesthetized with Equithesin (3.4 ml/kg) and a 21-g cannula (Plastics One, Roanoke, VA) was placed stereotaxically into the third ventricle using a previously described method (16, 25).

**Recombinant leptin synthesis and administration.** Recombinant human leptin was overexpressed in an *Escherichia coli* expression system and reconstituted to > 90% purity (see reference 5). The vehicle solution for icv administration to control rats was artificial CSF with a protein content matched to that in the leptin preparation. Icv injections (3.5  $\mu$ l) of either leptin (1.0  $\mu$ g/ $\mu$ l) or vehicle were performed over 1 min in lightly restrained, conscious rats. To determine its effect on hypothalamic neuropeptide gene expression, leptin was administered icv at both the onset and 16 h before the conclusion of a 40-h fast. To determine the effect of icv leptin on food intake, food was removed from ad libitum fed rats 1 h before icv leptin administration and 2 h before the onset of the dark cycle. Food was returned 15 min after icv infusion and food intake was quantified at 1, 4, and 24 h after icv injection.

### Assays and data analysis

**Protocol for *in situ* hybridization (ISH) to NPY and CRH mRNA.** Brains for ISH were immediately frozen on crushed dry ice, and subsequently sectioned at 14  $\mu$ m in a cryostat and mounted on RNAase-free slides. Hybridization was performed using  $^{33}$ P-labeled antisense oligonucleotide probes based upon cDNA sequences of rat CRH or NPY genes, as described elsewhere (16, 25). 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. Catharines, Ontario, Canada). The product of hybridization area (pixels) and density ( $\mu$ Ci/pixel) was used as an index of overall neuropeptide mRNA levels, since it correlates well with other measures of neuropeptide mRNA.

**Protocol for ISH to leptin receptor mRNA.** A 330-base antisense riboprobe was prepared from a linearized DNA template based on the sequence corresponding to the extracellular domain of the leptin receptor (26) (Genbank accession no. MMU42467) that was cleaved between bp2415 (EcoRI site) and bp2745 (XbaI site). Transcription of the riboprobe was done with T7 DNA-dependent RNA polymerase (Boehringer-Mannheim, Indianapolis, IN) in the presence of 25  $\mu$ M UTP containing  $^{33}$ P-UTP (Amersham, Arlington Heights, IL) at a ratio of 1:3 labeled to unlabeled UTP, for 1 h at 37°C, digested with DNAaseI, extracted with phenol-chloroform, and precipitated with ethanol. The pellet was dried and reconstituted in Tris-EDTA buffer to yield  $\sim$  2.1  $\mu$ g (19.3 pM), with 86% incorporation. Brains were obtained from adult (300 gram) male Wistar rats and handled as described above. The hybridization procedure (16) was carried out over-

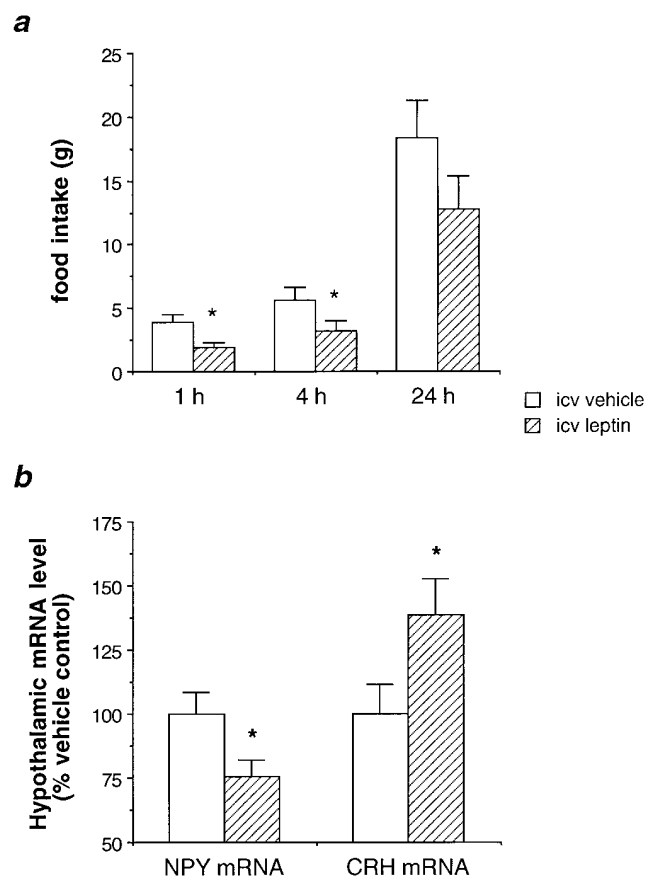
night in a moist chamber at 60°C, and subsequently, slides were placed in 2 $\times$  SSC buffer, transferred to RNase-containing buffer and washed for 30 min at 60°C. The slides were then washed in 0.1 $\times$  SSC at 22°C, dehydrated in ethanol, air-dried, and apposed to Hyperfilm BetaMax autoradiography film (Amersham, Arlington Heights, IL) for 5 d.

**Plasma assays.** Trunk blood was collected in EDTA-containing tubes at the time of sacrifice and removal of brain tissue (0700–0900 h) and placed on ice until plasma separation. Radioimmunoassays were used to measure plasma levels of corticosterone and immunoreactive insulin (IRI) as previously described (16, 27). Plasma glucose was determined by the glucose oxidase method (Beckman Instruments, Brea, CA).

**Statistical analysis.** Data are presented as group mean values ( $\pm$ SEM). Comparisons were performed with an unpaired, two-tailed Student's *t* test, with  $P < 0.05$  being considered sufficient to reject the null hypothesis of no difference between groups.

## Results

The effect of icv leptin on food intake in male Long-Evans rats is shown in Fig. 1 *a*. Food intake was measured 1, 4, and 24 h



**Figure 1.** (a) Food intake measured during the 24-h period after a single icv injection of either 3.5  $\mu$ g recombinant human leptin or vehicle in male Long-Evans rats ( $n = 12$ /group). (b) Effect of two icv injections of recombinant human leptin or vehicle in Long-Evans rats fasted for 40 h ( $n = 12$ /group) on levels of NPY and CRH mRNA, as determined by *in situ* hybridization. Leptin (3.5  $\mu$ g) or vehicle was administered as an icv injection at the onset and 16 h before the conclusion of a 40 h fast. \* $P < 0.05$  vs. icv vehicle control by unpaired two-tailed *t* test.

**Table I. Effect of icv Leptin Administration on Mean ( $\pm$ SE) Plasma Levels of Corticosterone, Glucose and Insulin in Fasted Long-Evans Rats\***

n	Treatment	Corticosterone	Glucose	IRI
		$\mu\text{g/dl}$	$\text{mg/dl}$	$\mu\text{U/ml}$
14	icv leptin	6.2 $\pm$ 2.0	121 $\pm$ 3	25.8 $\pm$ 4.3
13	icv vehicle	5.0 $\pm$ 1.5	118 $\pm$ 4	19.3 $\pm$ 2.7

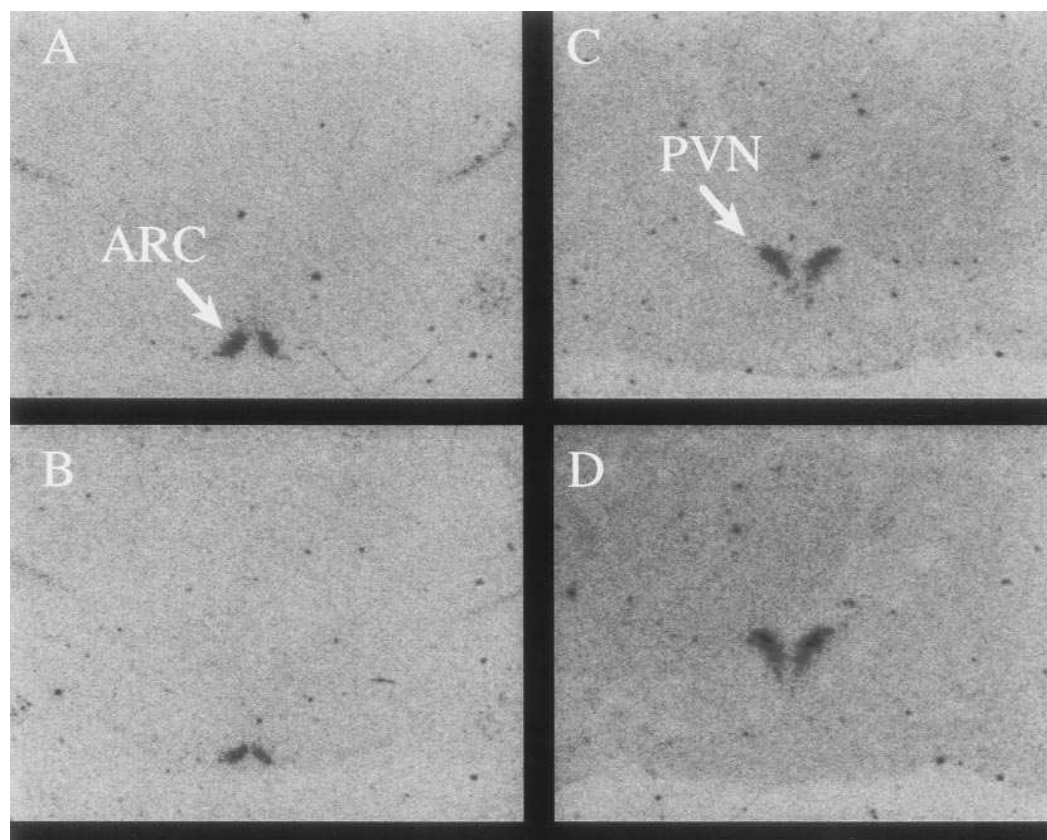
\*Blood samples were obtained from animals in which NPY and CRH mRNA determinations were also made. There were no significant differences between values from icv leptin vs. icv vehicle groups using an unpaired, two-tailed *t* test.

after a single icv injection of leptin (3.5  $\mu\text{g}$ ) at the onset of nocturnal feeding. Compared to an artificial CSF vehicle, icv leptin suppressed food intake by 50% over the first hour and 42% over the first 4 h (both  $P < 0.05$ ). Cumulative food intake over the 24-h period after icv leptin injection was reduced by 30% compared with controls, but this difference was not statistically significant. By comparison, intraperitoneal administration of the same dose of leptin did not significantly alter food intake at either the 1, 4, or 24 h time points (data not shown).

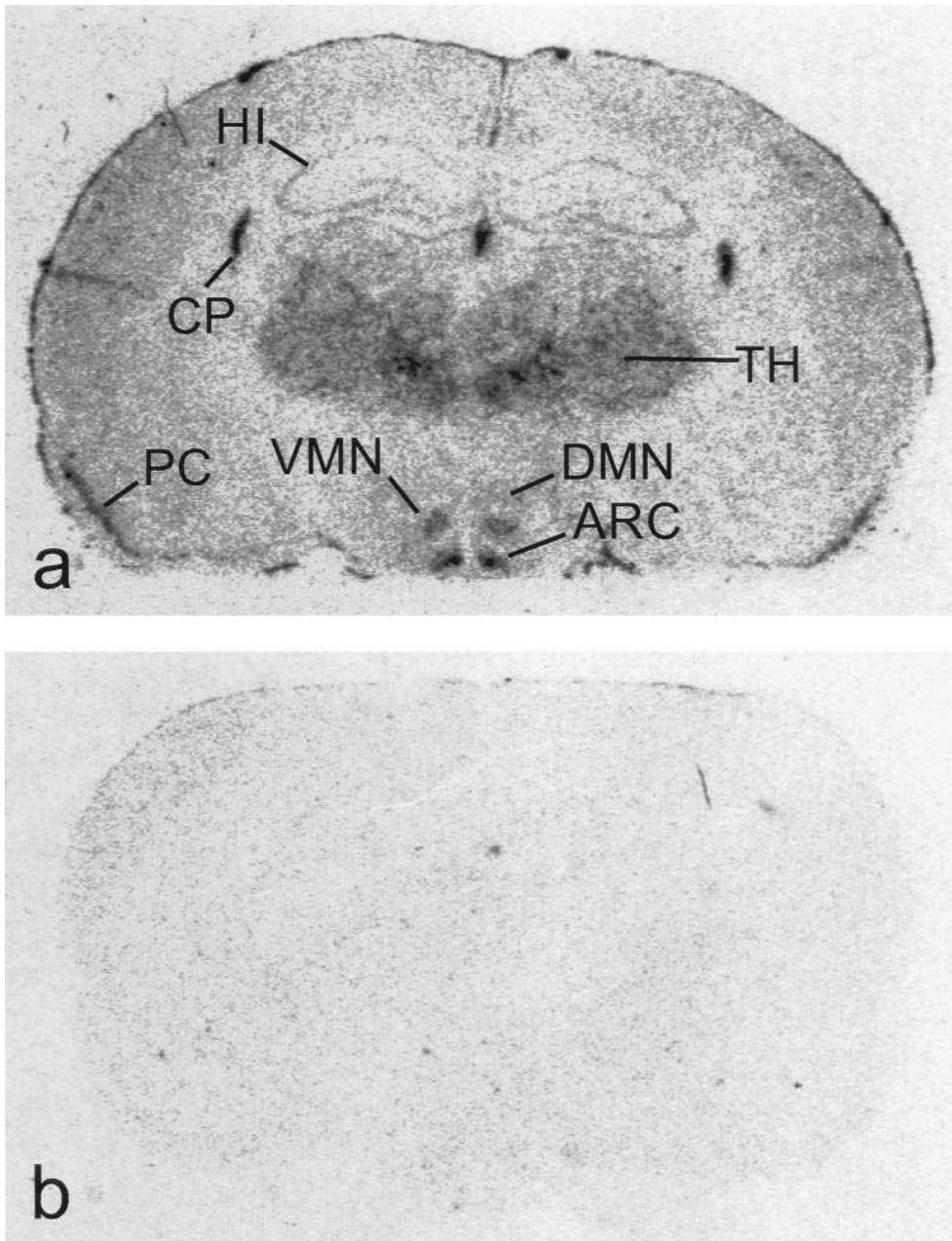
To determine the effect of leptin on hypothalamic neuropeptide gene expression in Long-Evans rats, we used in situ hybridization to measure levels of mRNA for NPY and CRH following icv leptin treatment (two injections of either leptin [3.5  $\mu\text{g}$ ] or vehicle during a 40-h period of food deprivation). As

expected, hybridization to mRNA encoding NPY was detected in the hypothalamic arcuate nucleus and in extrahypothalamic areas including cerebral cortex, hippocampus and the zona incerta of the thalamus of both leptin- and vehicle-treated animals. However, NPY mRNA levels were significantly lower in the arcuate nucleus of leptin-treated animals than in controls ( $-24\%$ ;  $P < 0.05$ ) (Figs. 1 *b* and 2, *A* and *B*). Hybridization to CRH mRNA was detected primarily in the hypothalamic PVN in these animals (Fig. 2, *C* and *D*). In the leptin-treated group, CRH mRNA levels in this brain area were increased by 38% as compared to vehicle-treated controls ( $P < 0.05$ ) (Fig. 1 *b*). Thus, whereas icv leptin reduced arcuate nucleus NPY mRNA levels, CRH mRNA levels were increased in the PVN of the same animals. To determine whether the effect of central leptin administration to increase CRH mRNA levels could have resulted from a response to nonspecific stress or toxicity associated with icv leptin administration, we also measured plasma levels of the adrenal glucocorticoid hormone, corticosterone. In contrast to its effect on CRH mRNA, icv leptin administration did not raise plasma levels of corticosterone, nor did it alter plasma glucose and insulin levels (Table I).

To determine if normal leptin receptors are necessary for the effect of icv leptin to alter hypothalamic NPY and CRH mRNA levels, we injected leptin or vehicle icv in obese Zucker (*fa/fa*) rats ( $n = 5/\text{group}$ ) fasted for 40 h using the same protocol that was employed in Long-Evans rats. In contrast to the reduction of NPY mRNA levels observed in Long-Evans rats, icv leptin administration caused a nonsignificant increase of 29% in the level of arcuate nucleus NPY mRNA in obese *fa/fa* Zucker rats as compared with icv vehicle-treated *fa/fa* controls



**Figure 2.** In situ hybridization to NPY (*A* and *B*) and CRH (*C* and *D*) mRNA in the hypothalamus of rats receiving icv vehicle (*A* and *C*) or leptin (*B* and *D*), according to the protocol described in Fig. 1. Autoradiograms were obtained from hybridizations performed on coronal sections of rat brain at the level of the midregion of the hypothalamus for NPY, and  $\sim 0.8$  mm rostrally, and at the caudal aspect of the optic chiasm for CRH mRNA. ARC, arcuate nucleus; PVN, paraventricular nucleus.



**Figure 3.** In situ hybridization to leptin receptor mRNA in rat brain. (a) Autoradiogram of leptin receptor mRNA hybridization in a coronal section of rat brain at the level of the midregion of the hypothalamus. *ARC*, arcuate nucleus; *VMN*, ventromedial hypothalamic nucleus; *DMN*, dorsomedial hypothalamic nucleus; *CP*, choroid plexus; *HI*, hippocampus; *TH*, thalamus; *PC*, pyriform cortex (b). Preincubation with RNase prevents hybridization.

( $P = 0.36$ ). Similarly, icv leptin administration increased nonsignificantly the level of CRH mRNA in the PVN of obese *fa/fa* rats as compared with vehicle-treated *fa/fa* controls ( $P = 0.40$ ).

To identify potential substrates for leptin action, we examined leptin receptor mRNA expression in rat brain by in situ hybridization, using a riboprobe complementary to mRNA encoding the extracellular domain of the receptor (designed to hybridize to all splice variants of the leptin receptor mRNA transcript). In the hypothalamus, hybridization for leptin receptor mRNA was densely concentrated in the arcuate nucleus, with lower levels detected in the ventromedial and dorsomedial nuclei (Fig. 3 a). Outside the hypothalamus, leptin receptor mRNA hybridization was also concentrated in choroid plexus and in the pyriform cortex, with somewhat lower levels detected in cerebral cortex, thalamus and hippocampus (Fig. 3 a). The specificity of this hybridization was demon-

strated by preincubation of brain sections with RNase, which completely abolished the hybridization signal (Fig. 3 b).

## Discussion

In the current study, we observed a potent and sustained anorexia induced by administration of leptin into the third cerebral ventricle of normal rats. Since intraperitoneal injection of the same dose of leptin did not alter food intake, these data suggest that leptin acts directly in the brain to reduce food intake, consistent with its proposed role in the negative feedback regulation of body adiposity. Moreover, this observation extends to normal animals the finding that food intake is potently suppressed following icv leptin administration in leptin-deficient, *ob/ob* mice (5). Using a similar approach, we recently

demonstrated that icv leptin reduces food intake not only under ad libitum feeding conditions, but also potently inhibits the hyperphagic response elicited by fasting, leading to a delayed recovery of lost weight (Seeley, R.J., et al. manuscript submitted for publication). Combined with the observation that fasting lowers the circulating leptin level (8), these results suggest that reduced leptin signaling in the brain may be an important component of the response to fasting that ensures the recovery of depleted fuel stores (18). If so, leptin administration into the brain of fasted animals should also attenuate specific hypothalamic responses to fasting.

Our data provide direct support for this hypothesis. We observed a significant reduction in the level of arcuate nucleus NPY mRNA and an increase in the level of CRH mRNA in the PVN of rats that received icv leptin during a 40-h period of fasting. Thus, both the decrease of hypothalamic CRH gene expression and the concomitant stimulation of NPY gene expression and feeding behavior induced by fasting (17, 19) appear to be mediated in part by reduced leptin levels in the brain, since leptin administration into brain ventricles attenuated these responses. Alternatively, these changes in neuropeptide mRNA level could reflect a nonspecific response to stress associated with icv leptin administration. However, this possibility seems unlikely since corticosterone levels were not altered by icv leptin administration in these animals. Moreover, leptin did not significantly alter NPY or CRH mRNA levels in the hypothalamus of fasted obese Zucker rats. Since obesity in these animals results from mutation of the leptin receptor (*fa/fa*) (3), our data suggest that normal leptin receptor function is required for the effect of icv leptin to influence hypothalamic NPY and CRH gene expression. These results are similar to those of previous studies demonstrating that icv administration of insulin lowers arcuate nucleus NPY mRNA levels during fasting in normal rats, but not in obese *fa/fa* rats (16, 28). Insulin and leptin, therefore, both appear to contribute to the regulation of hypothalamic NPY gene expression during fasting.

An additional finding from this study was the localization of leptin receptor gene expression to discrete areas of rat brain. While previous studies identified leptin receptor mRNA in extracts of choroid plexus and hypothalamus using reverse transcriptase PCR (26, 2), our use of *in situ* hybridization permitted us to investigate the regional pattern of leptin receptor gene expression in normal rat brain. We found that leptin receptor mRNA was highly concentrated in the arcuate nucleus, and was also detected in other areas implicated in the control of energy homeostasis (i.e., the ventromedial and dorsomedial hypothalamic nuclei). Combined with the effect of leptin to reduce levels of NPY mRNA in this hypothalamic area, our data suggests that the arcuate nucleus is a likely target of leptin action. In contrast, hybridization for leptin receptor mRNA was not detected in the hypothalamic PVN. Leptin stimulation of CRH mRNA expression in this brain area, therefore, appears to be mediated indirectly.

The probe used for this initial characterization of CNS leptin receptor gene expression was designed to hybridize to all known splice variants of leptin receptor mRNA, including those transcripts that encode the full-length intracellular domain (proposed to be competent for intracellular signal transduction) and those encoding the truncated intracellular domain (2). Additional studies are needed to determine the relative abundance and distribution of different leptin receptor

variants and to identify cell signaling systems activated by leptin in discrete neuronal populations the hypothalamus.

The concept that leptin acts on multiple hypothalamic targets is consistent with the proposed role of this hormone as an important physiological signal in the control of energy balance. While the role of other hypothalamic signaling systems (e.g., monoamines and/or peptides such as galanin, glucagon-like peptide-1, and melanin concentrating hormone) (29–31) await further study, we propose that leptin exerts its anorexic effects via a coordinated response that combines inhibition of NPY with stimulation of CRH-producing neurons. During fasting, therefore, reduced hypothalamic leptin signaling is proposed to stimulate NPY and to inhibit CRH production and release, a combination of responses that may contribute to the increased food intake and reduced energy expenditure essential for recovery of depleted fuel stores. The identification of NPY- and CRH-containing neurons as potential mediators leptin action, therefore, represents an important step towards an improved understanding of leptin's role in energy homeostasis in normal animals.

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