

# Increased Expression of mRNA for the Long Form of the Leptin Receptor in the Hypothalamus Is Associated With Leptin Hypersensitivity and Fasting

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The responsiveness of the hypothalamus to the inhibitory effects of leptin on food intake and body weight is influenced by multiple factors, including deficiency of either leptin or leptin receptors (Ob-R). To investigate whether altered expression of Ob-R in the hypothalamus could potentially contribute to altered leptin sensitivity, we performed *in situ* hybridization with riboprobes that detected either mRNAs encoding both the long (Ob-Rb) and short (Ob-Ra) splice variants or mRNA encoding only Ob-Rb. In the arcuate nucleus, mRNA encoding Ob-Rb, the predominant signaling form of the receptor, was 2.3 times greater in obese *db/db* and *ob/ob* mice than in lean *+/ob* controls ( $P < 0.01$ ). In *ob/ob* mice, systemic administration of leptin reduced Ob-Rb mRNA content of the arcuate nucleus by 30% compared with saline-treated, pair-fed controls ( $P < 0.05$ ). A 48-h fast increased Ob-Rb mRNA levels in the arcuate nucleus of normal and neuropeptide Y (NPY)-knockout mice ( $P < 0.01$ ), although the effect was greater in the NPY-knockout mice (400 vs. 247%,  $P < 0.05$ ). In addition, Ob-Rb mRNA hybridization was elevated by 40% in the arcuate nucleus ( $P < 0.05$ ) and by 75% in the ventromedial nucleus ( $P < 0.05$ ) of rats fasted 48 h. The results suggest that expression of Ob-Rb mRNA in the hypothalamus is sensitive to genetic and physiological interventions that alter circulating leptin levels, and that overexpression of Ob-Rb in the hypothalamus may contribute to increased leptin sensitivity. *Diabetes* 47:538–543, 1998

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ANOVA, analysis of variance; NPY, neuropeptide Y; NPY<sup>+/+</sup>, wild-type; NPY<sup>-/-</sup>, neuropeptide Y-deficient; Ob-R, leptin receptor; Ob-Ra, short-form splice variant of leptin receptor; Ob-Rb, long-form splice variant of leptin receptor; RT-PCR, reverse transcriptase-polymerase chain reaction.

The adipose tissue hormone, leptin (Ob protein), regulates adipose tissue mass by interacting with brain neurons that influence energy balance (1–4), and altered sensitivity of the brain to the anorexic effects of leptin is widely considered to be a causal factor in some forms of obesity (5). Systemic administration of leptin to rodents reduces food intake and body weight (1,6–9); this anorexic effect can be reproduced by chronic infusion of much lower doses of leptin directly into the hypothalamus (1,10–12). Recent studies indicate, however, that the responsiveness of the hypothalamus to the anorexic effect of leptin is variable and may be influenced by altered leptin signaling to the brain. For example, leptin sensitivity is increased in obesity that results from genetic deficiency of leptin (6,8,13,14) and is attenuated in obesity that is associated with defective leptin receptors (11,14,15). Mice with obesity resulting from dietary manipulations have high leptin levels and are relatively insensitive to leptin (1,4,12). Furthermore, leptin sensitivity is also elevated in mice lacking neuropeptide Y (NPY) (16) and can be modified by melanocortin receptor signaling in the brain (17).

We hypothesized that the brain's sensitivity to leptin is influenced by expression of leptin receptors (Ob-R) in the arcuate nucleus of the hypothalamus. The arcuate nucleus contains mRNA encoding the long-form splice variant (Ob-Rb) (18–20), which is considered to be the principal signaling Ob-R isoform in the brain (13,21–24). Recent immunocytochemical evidence that Ob Rb protein is also present in arcuate nucleus neurons (25) is consistent with mounting evidence that the arcuate nucleus is an important site for leptin's interaction with neurons involved in energy balance. Indeed, leptin suppresses the expression of mRNA encoding prepro-NPY (10,14,26–28) and increases pro-opiomelanocortin mRNA levels (29,41) in the arcuate nucleus. We hypothesized, therefore, that overexpression of Ob-Rb mRNA in the arcuate nucleus may contribute to the leptin hypersensitivity of mice deficient in leptin (*ob/ob* mice) (8) or NPY (NPY-knockout mice) (9).

To evaluate this hypothesis, we performed *in situ* hybridization with a riboprobe that recognizes mRNA encoding all of the known transmembrane splice variants of the murine Ob-R (total Ob-R mRNA). These variants include the short form (Ob-Ra), which is abundant in the choroid plexus

(30,31). Our results suggested that total Ob-R mRNA is over-expressed in the arcuate nucleus under conditions of low leptin signaling that results from the absence of either leptin (*ob/ob* mice) or functional leptin receptors (*db/db* mice), and that expression of Ob-R mRNA is reduced in the arcuate nucleus by systemic administration of leptin. We then performed *in situ* hybridization with a riboprobe that hybridizes specifically to Ob-Rb mRNA to determine whether Ob-Rb is the regulated form of the leptin receptor in the arcuate nucleus. We asked whether the leptin hypersensitivity that is exhibited by *ob/ob* mice (8) and by mice lacking the NPY gene (9) is associated with elevated expression of mRNA encoding Ob Rb in the arcuate nucleus. Finally, we asked whether Ob-Rb mRNA levels in the arcuate nucleus are physiologically regulated during fasting.

## RESEARCH DESIGN AND METHODS

**Animals.** Obese male C57Bl/6J *ob/ob* and C57Bl/6Ks *db/db* mice, lean *+ob* mice (littermates to the *ob/ob* mice), male NPY-deficient (NPY<sup>-/-</sup>) mice and their wild-type (NPY<sup>+/+</sup>) littermates, and pathogen-free male Wistar rats (280–300 g) were housed individually and maintained on a 12/12 h day/night cycle with ad libitum access to standard rodent diet and water, unless otherwise noted. Animals were killed by decapitation under CO<sub>2</sub> inhalation. All procedures met institutional guidelines for animal research and welfare.

**Leptin treatment of *ob/ob* mice.** Brain sections of *ob/ob* mice in the leptin treatment experiment came from a study that demonstrated a specific action of systemic leptin that caused reduced food intake, body weight, and NPY mRNA levels in the arcuate nucleus (14). The mice received five daily intraperitoneal injections of saline ( $n = 8$ ) or 150  $\mu$ g of recombinant murine leptin ( $n = 7$ ), with ad libitum feeding. A pair-fed control group was established by assigning each pair-fed *ob/ob* mouse ( $n = 8$ ) to a partner in the leptin treatment group. On each day, each pair-fed mouse received the amount of food consumed by its leptin-treated partner during the previous 24 h. The pair-fed mice received intraperitoneal injections of saline daily.

**Fasting studies.** The effect of fasting on Ob-Rb mRNA expression was evaluated in normal and NPY-deficient (NPY-knockout) mice. Production of the NPY-knockout mice has been previously described (9). Mice homozygous for the mutant NPY allele (NPY<sup>-/-</sup>) (27.5  $\pm$  1.1 g) and their normal littermates (NPY<sup>+/+</sup>) (27.2  $\pm$  1.0 g) were fed ad libitum ( $n = 8$ ) or deprived of food for 48 h ( $n = 12$ ). Body weights after food deprivation were similar for NPY<sup>-/-</sup> (20.3  $\pm$  0.6 g) and NPY<sup>+/+</sup> (19.5  $\pm$  0.5 g) mice. Plasma leptin levels for these mice have been previously reported (32). To assess the effect of fasting on Ob-Rb mRNA levels in the rat arcuate nucleus, rats ( $n = 8$ ) were fasted for 48 h, with water available ad libitum. Controls ( $n = 8$ ) had free access to food and water.

**Probes for *in situ* hybridization.** Riboprobes for *in situ* hybridization were prepared from cDNA clones that were obtained by reverse transcriptase-polymerase chain reaction (RT-PCR) of mouse and rat brain total RNA. A 330-base antisense riboprobe was prepared from a linearized DNA template based on the sequence of the murine leptin receptor (Genbank accession no. U42467) (31). The riboprobe recognizes the encoding regions for a portion of the extracellular domain and the entire transmembrane and intracellular domains of murine Ob-Ra splice variant mRNA (bases 2415–2745) and is complementary to all known transcripts that encode transmembrane forms of the leptin receptor, including the mutant leptin receptor mRNA variant found in the *db/db* mouse (30,33,34). A second antisense riboprobe of 568 bases that recognizes only Ob-Rb transcripts of mice was prepared from a cDNA clone (based on Genbank accession no. U46135) (34). This riboprobe corresponds to base positions 3040–3608, which are downstream from the alternative splice site and therefore not present in the transcripts for the Ob-Ra form. This Ob-Rb riboprobe is predicted to hybridize with the mutant Ob-Rb mRNA transcripts in the *db/db* mouse (34). For studies on rat brain, we cloned by RT-PCR a 352-base cDNA that is specific for the mRNA encoding the C terminus (bases 2899–3251) of Ob-Rb mRNA of rats (Genbank accession no. U52966) (15).

***In situ* hybridization.** Brains were removed immediately after decapitation between 0800 and 1200, frozen on dry ice, sectioned at 14  $\mu$ m with a cryostat, and mounted on RNase-free slides, followed by 4% paraformaldehyde, acetic anhydride, ethanol, and chloroform (35). The riboprobes were transcribed with T7 DNA-dependent RNA polymerase (Boehringer-Mannheim, Indianapolis, IN) in the presence of 25  $\mu$ mol/l UTP containing [<sup>32</sup>P]UTP (Amersham, Arlington Heights, IL) at a labeled-to-unlabeled ratio of 1:3 for 1 h at 37°C, then digested with DNase I, extracted with phenol-chloroform, and precipitated with ethanol. The pellets were dried, reconstituted in Tris-EDTA buffer, and used for *in situ* hybridization

following standard procedures (36). *In situ* hybridization with all probes was abolished when brain slices were pretreated with RNase. For each animal, four to six full coronal brain slices were used from the region of the hypothalamus that included the arcuate nucleus and ventromedial nucleus. All brain slices were concurrently prepared for hybridization, run in the same assay, and autoradiographed on film for 5 days (10).

**Data analysis.** Hybridization signals were quantified by calibrated computer densitometry of film autoradiographs as previously described (35,36). Analyses of variance (ANOVAs) for differences within and between groups were done with the multifactorial Scheffé's test. 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, with  $n$  representing animals per group.

## RESULTS

*In situ* hybridization of brain sections from *ob/ob*, *db/db*, and control mice with the riboprobe that recognized both Ob-Rb and Ob-Ra splice variant mRNA produced dense autoradiographic signals in the arcuate nucleus of all three genotypes, although the arcuate nucleus hybridization was visibly denser in *ob/ob* and *db/db* mice (Fig. 1A–C). Hybridization to total leptin receptor mRNA (Ob-Ra and Ob-Rb) in the arcuate nucleus of the obese *ob/ob* ( $n = 6$ ) and *db/db* ( $n = 6$ ) mice was 2.3-fold greater than that in the control lean *+ob* ( $n = 6$ ) mice (Scheffé's test,  $P < 0.01$ ) (Fig. 1D). Total Ob-R mRNA hybridization was similar in the choroid plexus of all genotypes and comparable to the elevated levels found in the arcuate nucleus of *ob/ob* and *db/db* genotypes. In *+ob* mice, Ob-R mRNA hybridization of the choroid plexus was 73% greater ( $P < 0.01$ ) than that of the arcuate nucleus.

Administration of leptin to *ob/ob* mice resulted in 30% lower total Ob-R mRNA hybridization in the arcuate nucleus compared with administration of the saline vehicle to pair-fed control mice (with the same food intake as the leptin-treated mice) (Fig. 2A). This effect was significant by ANOVA across all groups (Scheffé's test,  $P = 0.0118$ ), and by comparison of the leptin-treated and pair-fed groups (Scheffé's test,  $P = 0.0125$ ). Control mice that received saline and were fed ad libitum also had a higher mean value for total Ob-R mRNA hybridization in the arcuate nucleus compared with the leptin-treated mice, but the difference was not significant.

To test the hypothesis that the Ob-Rb splice variant is the principal regulated leptin receptor form in the arcuate nucleus, we conducted *in situ* hybridization with the riboprobe that recognizes only Ob-Rb variant mRNA. With this riboprobe, the hybridization signal for Ob-Rb mRNA in the arcuate nucleus versus that in the choroid plexus was 2.3 times higher in the lean *+ob* mice and 3.7 times higher in the *ob/ob* and *db/db* obese mice, respectively (Scheffé's test,  $P < 0.001$ ). Furthermore, hybridization for Ob-Rb mRNA in the arcuate nucleus was elevated twofold in the obese *ob/ob* and *db/db* mice compared with the lean *+ob* controls (Scheffé's test,  $P < 0.01$ ) (Fig. 2B), similar to the results for total leptin receptor mRNA (compare Figs. 2B and 1D). The reduction of the choroid plexus hybridization signal when only Ob-Rb mRNA was detected, compared with the signal when both Ob-Ra and Ob-Rb mRNA were detected, was visually apparent (Fig. 2C and D). Thus, genetic leptin deficiency (*ob/ob*) and leptin resistance (*db/db*) were associated with overexpression of the Ob-Rb splice variant mRNA in the arcuate nucleus.

The visible density of hybridization for Ob-Rb mRNA in the arcuate nucleus of both normal (NPY<sup>+/+</sup>) and NPY-deficient (NPY<sup>-/-</sup>) mice (Fig. 3A and B) was increased by fasting (Fig. 3C and D). The elevated expression of Ob-Rb mRNA was

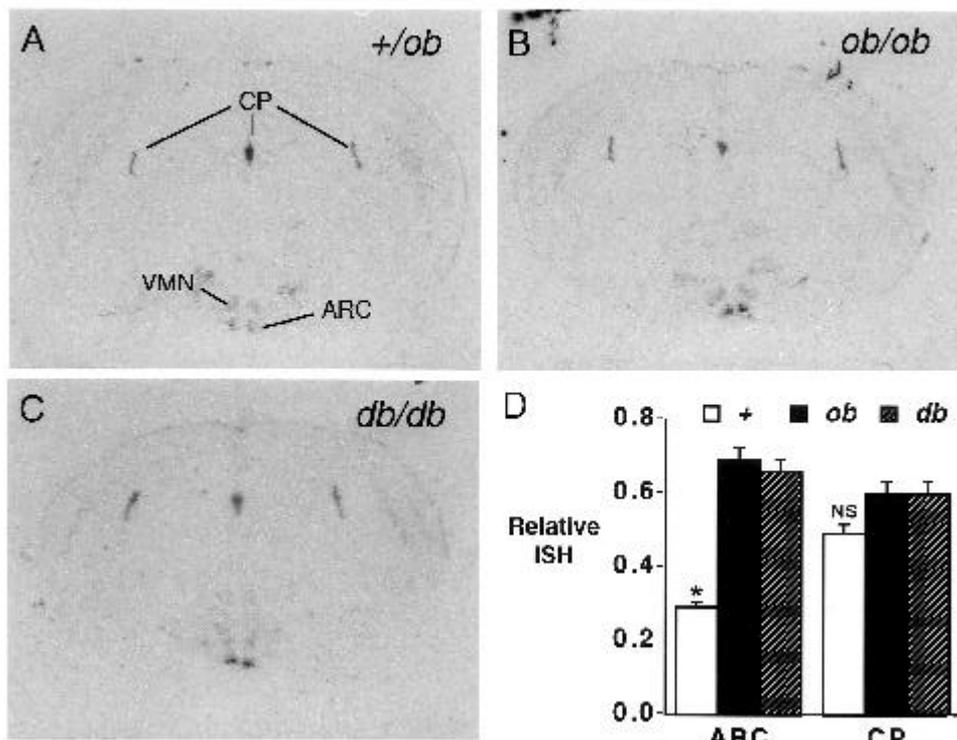


FIG. 1. In situ hybridization with riboprobe that recognizes mRNA for both Ob-Rb and Ob-Ra splice variants, showing hybridization in choroid plexus (CP), arcuate nucleus (ARC), and ventromedial nucleus (VMN) from the brains of lean (+/ob) (A), ob/ob (B), and db/db (C) mice. D: The relative in situ hybridization (ISH) signals in choroid plexus and arcuate nucleus of lean (+), ob/ob (ob), and db/db (db) mouse brains. \* $P < 0.01$ , +/ob vs. ob/ob and db/db arcuate nucleus;  $P < 0.001$ , choroid plexus vs. arcuate nucleus. NS,  $P > 0.05$  for +/ob vs. ob/ob and db/db choroid plexus and for choroid plexus vs. arcuate nucleus.

significantly greater ( $t$  test,  $P < 0.05$ ) in the NPY-deficient mice than in normal controls (400 vs. 247%), and the effect of fasting was significant for both genotypes ( $t$  test,  $P < 0.01$ ) (Fig. 3E). There were no differences in Ob-Rb hybridization among fed mice. Although dense hybridization was present in the ventromedial nucleus, there was no measurable difference between genotypes or treatments. Thus, fasting for 48 h resulted in large increases of arcuate nucleus Ob-Rb mRNA levels in normal mice, and the effect was amplified in the absence of NPY.

We also measured Ob-Rb mRNA levels in the arcuate nucleus of fed and fasted rats. As in mice, fasting increased hybridization for Ob-Rb mRNA in the arcuate nucleus of the rat hypothalamus, although the effect appeared less robust than in mice (Fig. 4A and B). Increased hybridization signal was also seen in the ventromedial nucleus of the rats, an effect not seen in mice. In the fasted rats, mRNA for Ob-Rb was increased by 40% ( $t$  test,  $P < 0.05$ ) in the arcuate nucleus and by 75% ( $t$  test,  $P < 0.05$ ) in the ventromedial nucleus, compared with the fed controls (Fig. 4C). There were no significant differences in Ob-Rb mRNA levels in the rat thalamus. Therefore, fasting was accompanied by increased Ob-Rb mRNA expression in the arcuate nucleus of both rat and mouse brain.

## DISCUSSION

To investigate the hypothesis that changes in the brain's sensitivity to leptin may involve altered expression of Ob-R in the arcuate nucleus, we performed in situ hybridization with two

riboprobes, one that detects only mRNA encoding Ob-Rb, the signaling form of the leptin receptor, and one that detects total Ob-R mRNA (Ob-Ra and Ob-Rb). The results show that total Ob-R mRNA is markedly elevated in the arcuate nucleus of ob/ob (leptin-deficient) mice compared with lean +/ob littermates and that Ob-Rb mRNA is the predominant overexpressed splice variant in the hypothalamus, which is consistent with recent reports (26,37). We reasoned that if Ob-Rb mRNA expression is stimulated by reduced leptin signaling, then arcuate nucleus levels of Ob-Rb transcripts should also be elevated in the db/db mouse, which synthesizes leptin but is insensitive to the hormone because its Ob-Rb receptor is defective (14). The results show that db/db mice overexpress the mutant Ob-Rb mRNA in the arcuate nucleus at levels equivalent to the overexpressed normal Ob-Rb transcripts in ob/ob mice. Therefore, while the results do not rule out contributions of other components of the obesity syndrome to Ob-Rb mRNA expression, overexpression of Ob-Rb mRNA in the arcuate nucleus of ob/ob and db/db mice is likely a consequence of reduced leptin signaling.

In contrast, Ob-R mRNA levels in the arcuate nucleus were lowered by administration of leptin to ob/ob mice. Pharmacological doses of leptin were recently reported to decrease Ob-R mRNA in the arcuate nucleus of the ob/ob mouse in a study that omitted the pair-fed group required to control for the anorexic effect of leptin (26). The present study included a vehicle-infused control group that was pair fed to the leptin-treated group. The results show that systemic leptin administration to leptin hypersensitive ob/ob mice reduces

FIG. 2. **A:** Relative in situ hybridization (ISH) of total leptin receptor mRNA in the arcuate nucleus of *ob/ob* mice fed ad libitum was reduced after systemic leptin treatment for 5 days (LEPTIN AD LIB) compared with controls that were fed ad libitum and received vehicle (VEHICLE AD LIB) and mice that were pair fed (VEHICLE PAIR) to the leptin-treated group. \* $P = 0.0125$ , leptin-treated group vs. pair-fed group. **B:** Relative in situ hybridization signals in choroid plexus (CP) and arcuate nucleus (ARC) of lean (+), *ob/ob* (*ob*), and *db/db* (*db*) mouse brains with riboprobe that recognizes mRNA encoding only Ob-Rb mRNA. \* $P < 0.01$ , +/*ob* vs. *ob/ob* and *db/db* arcuate nucleus. \* $P < 0.001$ , choroid plexus vs. arcuate nucleus; \* $P < 0.01$ , +/*ob* arcuate nucleus vs. choroid plexus. **C and D:** In situ hybridization autoradiographs of *ob/ob* brain, showing hybridization to choroid plexus and arcuate nucleus with probe for both Ob-Ra and Ob-Rb (**C**), and absence of hybridization in choroid plexus with probe specific for Ob-Rb (**D**).

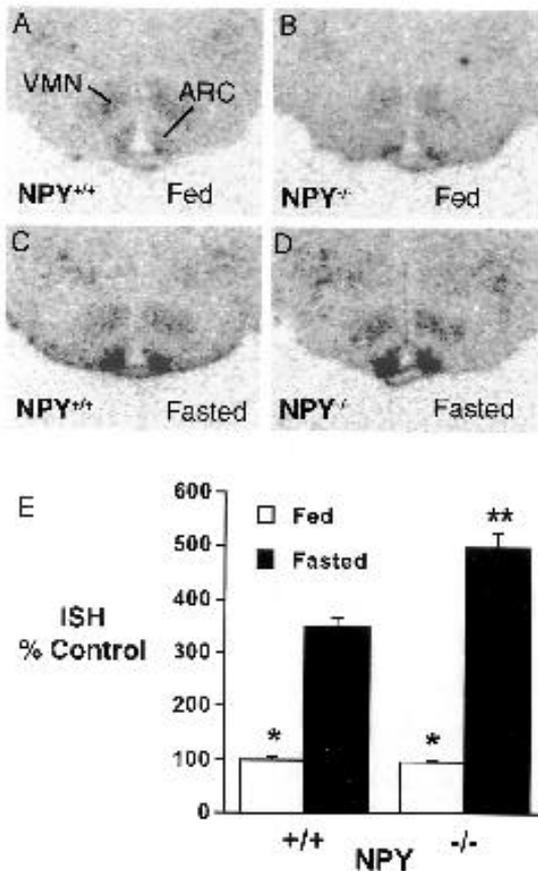
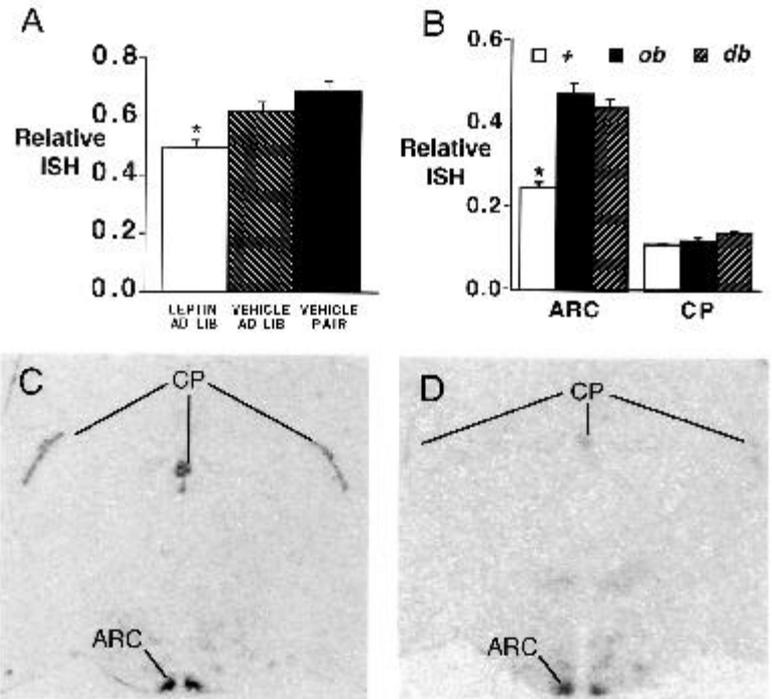


FIG. 3. In situ hybridization (ISH) for Ob-Rb mRNA in arcuate nucleus (ARC) and ventromedial nucleus (VMN) of fed (**A**) and fasted (**C**) normal control (*NPY*<sup>+/+</sup>) mice and fed (**B**) and fasted (**D**) *NPY*-knockout (*NPY*<sup>-/-</sup>) mice. **E:** Increased arcuate nucleus Ob-Rb mRNA in fasted normal mice (\* $P < 0.01$ ) and its amplification in fasted *NPY*-knockout mice (\*\* $P < 0.05$ ) compared with fasted controls.

total Ob-R mRNA levels in the arcuate nucleus when food intake is unchanged, which is consistent with the hypothesis that Ob-R expression in the brain is modulated by leptin signaling. Further studies are needed to determine whether Ob-R mRNA levels can be modified by leptin treatment in normal animals that produce leptin and do not overexpress Ob-R in the hypothalamus.

A major new finding of this study is the increase of Ob-Rb mRNA expression in the arcuate nucleus of rats and mice that resulted from a 48-h fast. This effect was greater in mice (247%) than in rats (40%), a difference that may be related to the proportionately greater weight loss experienced by mice (~27%) compared with rats (~13%) during a 48-h fast (10,11,14). The mechanism responsible for the increase of hypothalamic Ob-Rb mRNA levels during fasting could be related to low levels of circulating leptin (38,39). Interestingly, Ob-Rb mRNA levels in the arcuate nucleus of the leptin-hypersensitive *NPY*-deficient mice (*NPY*<sup>-/-</sup>) increased 400% compared with 247% in fasting normal control mice (*NPY*<sup>+/+</sup>). The augmentation of the fasting-induced increase in Ob-Rb mRNA levels in *NPY*<sup>-/-</sup> versus *NPY*<sup>+/+</sup> mice is probably not due to differences in circulating leptin levels, because the fasted *NPY*<sup>+/+</sup> and *NPY*<sup>-/-</sup> mice used in this study had similar plasma leptin levels (32). Furthermore, the augmented expression of Ob-Rb mRNA in the arcuate nucleus of the *NPY*-deficient mice during fasting compared with that in the normal mice supports the hypothesis that *NPY* signaling opposes the anorexic effects of leptin (9).

The findings of the present study indicate that expression of mRNA for Ob-Rb in the hypothalamus, particularly in the arcuate nucleus, is sensitive to physiological interventions that cause a change in circulating leptin levels. The finding that Ob-Rb mRNA was overexpressed in the arcuate nucleus of *ob/ob* mice and fasted *NPY*-deficient mice may explain their hypersensitivity to leptin (1,8,9). Furthermore, the data are compatible with the suggestion that Ob-Rb protein may be expressed by non-*NPY* neurons in the hypothalamus (40),

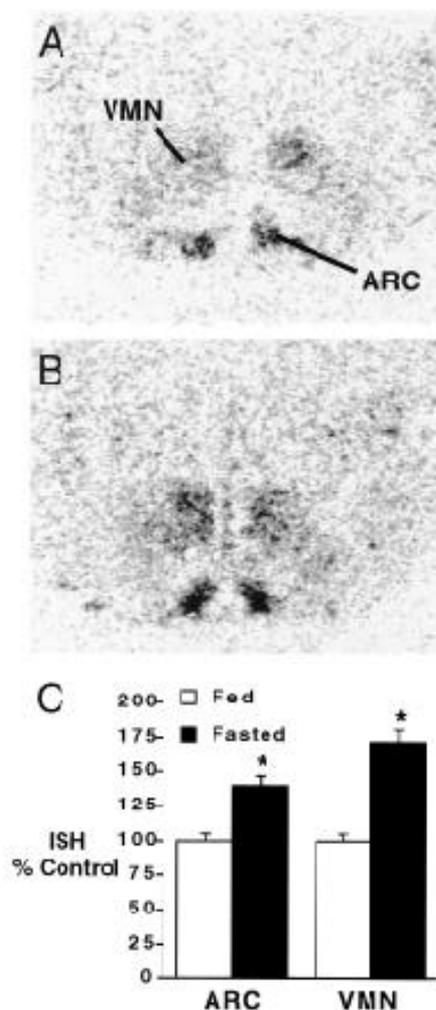


FIG. 4. In situ hybridization (ISH) of Ob-Rb mRNA in arcuate nucleus (ARC) and ventromedial nucleus (VMN) of rats that were fed (A) and of those fasted (B) for 48 h. C: In situ hybridization signal for Ob-Rb mRNA showing increased Ob-Rb mRNA content of arcuate nucleus and ventromedial nucleus of fasted rats compared with fed controls (\* $P < 0.05$ ).

which could explain why NPY-deficient mice have increased, rather than decreased, responsiveness to leptin. These data are consistent with the hypothesis that binding of leptin to Ob-Rb at the surface of arcuate nucleus neurons may initiate intracellular signal transduction events that result in reduced transcription of the Ob-Rb splice variant. Furthermore, the results indicate that the Ob-Rb splice variant is a major regulated form of the leptin receptor in the hypothalamus. These new findings indicate that altered expression of the leptin receptor gene is associated with physiological changes in circulating leptin, although the data cannot rule out the possibility that metabolic or hormonal conditions in addition to the hypoleptinemia of fasting may also contribute. The present results suggest, therefore, that regulation of Ob-Rb expression in the hypothalamus may be an important component of the brain's adaptive response to changes in energy balance.

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