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# Proopiomelanocortin Neurons Are Direct Targets for Leptin in the Hypothalamus

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**ABSTRACT**

Leptin is a protein product of the obese (*ob*) gene, which is secreted by adipocytes and functions as a satiety factor to regulate food intake. The expression of the leptin receptor in several hypothalamic nuclei suggests that multiple neuronal subtypes are targets for leptin's action. Products of the proopiomelanocortin (POMC) gene are known to affect feeding behavior, and POMC neurons share a similar distribution with leptin receptor mRNA in the arcuate nucleus. We used double label *in situ* hybridization and computerized image analysis to test the hypothesis that POMC neurons coexpress the leptin receptor. Quantitative analysis confirmed that POMC neurons in the hypothalamus express leptin receptor mRNA. Based on this observation, we infer that POMC neurons and the products of the POMC gene may be part of the signaling pathway mediating leptin's action on feeding and perhaps other physiological functions.

Leptin is a protein hormone that plays an important role in the regulation of body weight. Leptin circulates in concentrations that are proportional to the degree of body adiposity and acts as a satiety factor to regulate food intake and metabolism (1-4). Leptin has also been implicated as a metabolic gate linking nutrition and fertility (5-8). Since feeding behavior and many aspects of metabolism and reproduction are regulated by the brain, particularly the hypothalamus, neurons in this area are likely targets for the action of leptin. Indeed, leptin receptor (OB-R) mRNA is expressed in several hypothalamic regions, including the ventromedial, dorsomedial, and arcuate nuclei (9,10); however, the full identification of the target cells for leptin in the brain is just beginning. Likely candidates include neuropeptide Y (NPY) and proopiomelanocortin (POMC) neurons, which are thought to be involved in the control of feeding and reproduction (11-16).

NPY-expressing neurons are plausible targets for the action of leptin (10,17) and recent evidence indicates that NPY-containing neurons may express the leptin receptor (18, 19). A compelling case can be made for an important modulatory function for NPY in the control of reproduction and NPY neurons appear to be part of the signaling pathway that mediates leptin's effect on food intake (17,20), but whether NPY is *essential* in these processes has been challenged with the observation that mice with targeted mutations of the NPY gene exhibit relatively normal phenotypes with respect to their body weight regulation and fertility (21). Moreover, crossing NPY-deficient mice with obese mice (*ob/ob*), which lack a functional leptin gene and overexpress NPY, results in offspring that are still somewhat obese (22). These observations suggest that other factors, besides NPY, are involved in the mediation of leptin's action in the hypothalamus.

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POMC-expressing neurons in the arcuate nucleus are another possible target for leptin's action in the regulation of both feeding and reproduction. Short term food restriction can significantly attenuate POMC gene expression (12,13). Furthermore, two of the peptide products of the POMC gene, β-endorphin (βEND) and α-melanocyte-stimulating hormone (αMSH) have been implicated in the regulation of gonadotropin secretion and feeding behaviors (11-16,23). We tested the hypothesis that POMC neurons themselves are direct targets for the action of leptin by identifying POMC-containing neurons in the hypothalamus that coexpress leptin receptor mRNA.

## Materials and Methods

*Animals.* Adult female Sprague-Dawley rats (n=2) were purchased from Simonsen Laboratories (Gilroy, CA). They were housed in temperature- and light-controlled conditions (12:12 light/dark cycle with lights on at 0600 h) and were fed *ad libitum* on standard rodent chow. Animals were asphyxiated in carbon dioxide, decapitated and their brains were rapidly removed, frozen on dry ice, and stored at -80°C. Twenty-micron coronal brain sections were cut through the hypothalamus on a cryostat, thaw-mounted onto Superfrost Plus Microscope Slides (Fisher Scientific, Pittsburgh, PA), and stored at -80°C until *in situ* hybridization was conducted.

*OB-R riboprobe synthesis.* The plasmid vector containing a cDNA to mouse OB-R mRNA was generously provided by Dr. Joseph Kuijper (ZymoGenetics, Inc, Seattle, WA). An EcoRI/XbaI fragment (bases 2415 to 2750) that maps to the transmembrane domain of both the short and long OB-R form was subcloned into pBluescript II KS (Stratagene, La Jolla, CA). Antisense cRNA probe was synthesized using the Ambion MAXIscript kit (Ambion, Austin, TX) in the presence of $^{33}$P-UTP (Dupont NEN, Boston, MA).

*POMC riboprobe synthesis.* The plasmid vector containing a cDNA to mouse pituitary POMC precursor was kindly

provided by Dr. Michael Uhler. A 925-basepair HindIII-EcoRI fragment was subcloned into pSp64 (Promega Biotec, Madison, WI). Antisense digoxigenin-labeled POMC cRNA probe was synthesized with digoxigenin-labeled-rUTP (Boehringer Mannheim, Indianapolis, IN) and the Ambion MAXIscript kit.

*Double Label In Situ Hybridization.* We performed double-label *in situ* hybridization to identify cells containing both OB-R mRNA and POMC mRNA following a protocol described previously (24), with modification. Briefly, processed tissues were hybridized with riboprobe-hybridization buffer mix, which contained the $^{33}$P-labeled OB-R cRNA probe (~$2x10^8$ cpm/ml) and the digoxigenin-labeled POMC cRNA probe (optimal concentration for digoxigenin-labeled riboprobes was empirically determined to be 1:50). Overnight hybridization at 55°C was followed by RNase treatment, a series of stringent SSC washes, and a wash at 60°C. The slides were then blocked with 2% normal sheep serum, incubated with antidigoxigenin antibody conjugated to alkaline phosphatase (Boehringer Mannheim, Indianapolis, IN) at 37°C, and subjected to a chromagen reaction containing NBT and BCIP (Sigma, St. Louis, MO). All slides were then air-dried, dipped in 3% parlodion followed by NTB3 nuclear emulsion (Kodak, Rochester, NY) and exposed for 2 months at 4°C. All slides were processed, hybridized, exposed, and developed together.

*Image Analysis.* Tissue sections were analyzed according to a protocol published previously (25). Briefly, sections were viewed using a Zeiss Axioskop microscope (Zeiss, New York, NY) and a Dage model 85 camera (Dage-MTI, Inc, Michigan City, IN). Digoxigenin-labeled neurons were outlined under bright field illumination and silver grains were counted over the outlined neurons under darkfield illumination. Signal to background ratios (SBRs) were calculated as previously described (25).

*Statistical Analysis.* To ascertain whether POMC neurons expressed detectable amounts of leptin receptor, SBRs for all of the cells measured in an animal were averaged to obtain a mean SBR for each animal. A one-sample Student's t-test was then used to determine whether these mean SBR differed significantly from 1 (the value that would be expected if there was no specific signal).

## Results

POMC mRNA-containing neurons, represented by cell bodies with deep blue color products, were widely distributed throughout the arcuate nucleus and the retrochiasmatic area of the hypothalamus. Many POMC neurons had silver grain clusters lying directly over the cells, reflecting the presence of leptin receptor mRNA (Fig. 1). Automated image analysis showed that with a signal to background ratio of 3 as the criterion, approximately half of the digoxigenin-labeled POMC mRNA-containing neurons can be said to express leptin receptor mRNA (Table 1). The number of grains over the POMC neurons was significantly higher than background ($p<0.05$) (Fig. 2).

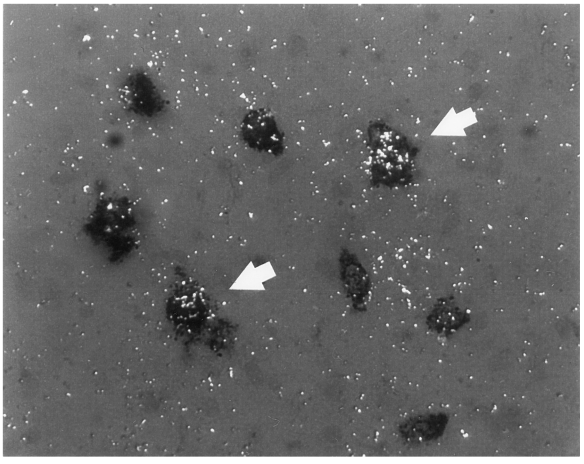

Fig. 1. Photomicrograph showing digoxigenen-labeled POMC neurons (darkly stained) in the arcuate nucleus. White silver grains mark the presence of leptin receptor mRNA. White arrows point to two cells that appear to express both POMC and leptin receptor mRNA. Whether the other POMC neurons in this picture express leptin receptor mRNA is less certain. This uncertainty underscores the importance of quantitative analysis.

**TABLE 1.** Percentage of double-labeled neurons under various signal to background ratio (SBR) criteria.

| SBR | Mean % of Double Labeled Neurons |
|---|---|
| 0 | 100 |
| 1 | 89.6 ± 3.2 |
| 2 | 72.2 ± 2.3 |
| 3 | 50.9 ± 3.2 |
| 4 | 32.8 ± 2.6 |

## Discussion

Considerable evidence suggests that POMC gene products are involved in the regulation of body weight and reproduction. A recent study in humans highlights the potential importance of the POMC gene itself in the regulation of body weight. Comuzzie and co-workers have reported that polymorphisms associated with obesity and leptin levels map near the POMC gene (26). Additional studies in rats have demonstrated that POMC gene products, acting within the central nervous system, influence both food intake and gonadotropin secretion (14,15,27) and that POMC neurons establish synaptic contacts with GnRH neurons (28,29). However, these earlier studies do not offer a clear explanation for how POMC neurons might integrate the control of reproduction and body weight. The observation that POMC neurons express the leptin receptor marks them as potentially key elements in mediating leptin's influence on feeding and reproduction.

We have observed that the percentage of POMC neurons that can be accepted as coexpressing the leptin receptor varies as a function of SBR. With a SBR criterion of 2, approximately 70% of POMC neurons would appear to express the leptin receptor, whereas at a SBR of 4, about 35% of these cells would be said to express the receptor. The idea that some, but not all POMC neurons may express the leptin

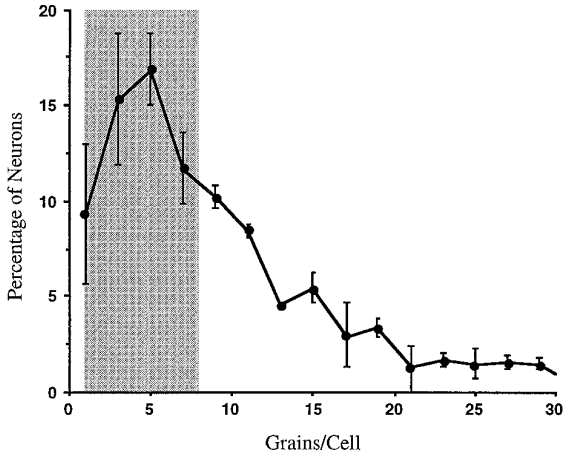


Fig. 2. Frequency histogram showing the percentage of POMC neurons expressing different grain counts (solid line) reflecting leptin receptor mRNA. The shaded area represents the 95% confidence limits of the background grain count estimates over nearby areas not containing POMC neurons.

receptor suggests that only a subset of the cells are directly involved in regulation of body weight by the action of leptin. Indeed, considerable evidence suggests that POMC neurons in the arcuate nucleus are a highly heterogeneous population involved in a myriad of physiological functions (30). However, considering the threshold-dependent nature of assigning a particular value for the percentage of cells co-expressing the receptor, it can't be ruled out that *all* POMC neurons express the leptin receptor. These variable determinations, reflecting the relative stringency of the acceptance criteria, underscore the importance of quantitative analysis of the double-labeling technique as well as its limitations.

There are several peptide products of the POMC gene that could be involved in leptin's action in areas of the brain that control metabolism and reproduction. One candidate is β-endorphin (βEND), which has been implicated in the regulation of food intake and gonadotropin secretion, based on several lines of evidence (11). First, βEND levels are elevated in *ob/ob* mice (31). Second, the central administration of βEND increases food intake and induces hyperglycemia in the rat (32). Third, βEND has a potent inhibitory effect on LH secretion, presumably by blocking the release of gonadotropin-releasing hormone (GnRH), whereas opiate receptor blocking agents accelerate the frequency of pulsatile GnRH secretion. The possible significance of βEND in leptin's signaling pathway in the hypothalamus is clouded by the fact that none of the classical opiate receptor subtypes (μ, κ and δ) appear to be expressed by GnRH neurons (33). In addition, βEND-deficient mice apparently have normal reproductive function (34).

Recent studies have revealed that αMSH, a non-opioid product of the POMC gene, may also be involved in mediating the interaction of POMC-containing neurons and GnRH neurons, and perhaps even leptin's role in bridging nutrition and reproduction. αMSH is synthesized by POMC neurons in the arcuate nucleus and binds to the MC4 receptor (MC4-R) (35). Mice with MC4-R inactivation have adult-onset obesity, and the administration of MC4-R agonists to normal mice results in decreased food intake similar to that seen with leptin treatment (16,23). Together, these observations suggest a working framework in which, as a derivative of POMC, αMSH tonically inhibits centrally mediated feeding activity and leptin stimulates αMSH expression, which in turn induces satiety. Can this idea be integrated with what's known about the effects of leptin and αMSH on the neuroendocrine reproductive axis? First, leptin exerts a powerful stimulatory effect on the reproductive axis (5-8), and second, αMSH stimulates the release of GnRH (14,15). Thus, it's at least conceivable that leptin's role in stimulating the reproductive axis and in promoting satiety are both mediated by αMSH. It will be interesting to identify the phenotypes of cells expressing the MC4-R and to determine whether GnRH neurons express this receptor—indeed, if this were the case, it would support the hypothesis that leptin's action on both feeding and reproduction are coordinated by αMSH. A recent study by Kesterson and co-workers, however, shows that POMC mRNA levels remain unchanged in both *ob/ob* mice and mice which have chronically antagonized MC4-R (36). This observation suggests that either the regulation of αMSH is post-translational or that non-POMC gene products in POMC neurons mediate leptin's signaling action.

In summary, we have identified POMC neurons in the arcuate nucleus as a probable target for leptin's action in the hypothalamus by demonstrating that these cells express leptin receptor mRNA. Based on the observations that the neuropeptide products of the POMC gene have been shown to participate in the regulation of food intake and gonadotropin secretion, we infer that at least some of the effects of leptin on feeding and reproduction may be mediated by POMC neurons.

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