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Regulation of Hypothalamic Proopiomelanocortin mRNA by Leptin in *ob/ob* Mice.

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ABSTRACT

The hormone leptin acts on the brain to regulate feeding, metabolism, and reproduction; however, its cellular targets and molecular mechanisms of action remain to be fully elucidated. The melanocortins, which are derived from the precursor proopiomelanocortin (POMC), are also implicated in the physiological regulation of body weight. POMC-containing neurons express the leptin receptor, and thus it is conceivable that the POMC gene itself may be part of the signaling pathway involved in leptin's action on the brain. Using *in situ* hybridization and computerized image analysis, we tested the hypothesis that the POMC gene is a target for regulation by leptin by comparing cellular levels of POMC mRNA in the hypothalamus among groups of leptin-deficient (*ob/ob*) mice, leptin-treated *ob/ob* mice, and wild-type controls. POMC mRNA levels were significantly reduced throughout the arcuate nucleus in vehicle-treated *ob/ob* mice relative to wild-type controls, whereas POMC mRNA levels in leptin-treated *ob/ob* mice were indistinguishable from wild-type controls. These observations suggest that one or more products of POMC serve as an integrative link between leptin and the central mechanisms governing body weight regulation and reproduction.

LEPTIN is a hormonal product of adipose tissue whose expression reflects the body's state of nutritional reserves (1, 2). Leptin is thought to act as a metabolic signal to the brain causing adjustments in feeding behavior and metabolism that allow the body to maintain normal weight (3-6). Mice that are genetically deficient in leptin (*ob/ob*) are hyperphagic, obese, and infertile, and the administration of leptin to these animals reverses their metabolic and reproductive dysfunction (3-8). Although evidence suggests that these effects of leptin on feeding and reproduction reflect its action on the brain (8, 9), neither its target cells nor its cellular mechanism of action is fully understood.

The leptin receptor is expressed in several hypothalamic nuclei that have been implicated in the regulation of metabolism and reproduction—including the ventromedial, paraventricular, and arcuate (ARC) nuclei (10-12). One candidate for mediating leptin's action in the hypothalamus is neuropeptide Y (NPY)—a potent orexigenic factor which has been experimentally linked to the neural control of feeding and reproduction (10, 13). A recent report suggests that NPY-containing neurons in the ARC express leptin receptor mRNA (14). The expression of NPY is dramatically increased in *ob/ob* mice, and this elevated level of NPY expression can be reduced by the administration of leptin (15). Although NPY appears to be an important element in the neuronal circuitry controlling these physiological functions, other recent studies suggest that NPY does not act alone, and that other neuropeptide systems also appear to be requisite (16, 17).

Based on several lines of evidence, proopiomelanocortin (POMC) has emerged as another candidate for mediating leptin's action in the hypothalamus. POMC is a precursor protein that is differentially processed in various neurons to produce a variety of peptides, including α-melanocyte-stimulating hormone (α-MSH) and β-endorphin (18), both of which have been implicated in the regulation of feeding, metabolism, and reproduction (10, 19, 20). Moreover, one of the melanocortin receptors, MC4 receptor (MC4-R), has been directly linked to the obesity syndrome found in the *agouti* mouse (20, 21). POMC neurons also appear to be a direct target for leptin's action—this is based on the recent discovery that POMC mRNA-containing neurons in the rat express leptin receptor mRNA (22). Together, these observations make a strong inferential case for the involvement of POMC neurons in the regulation of feeding and metabolism and implicate the POMC gene itself as a potential target for regulation by leptin.

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Materials and Methods

Animals. Adult (5 mo old) male *ob/ob* (n=10) and lean wild-type (n=5) mice from ZymoGenetics, Inc., were housed on a 12:12 light/dark cycle with lights off at 1800 h. Animals were housed in groups of 2-5 and allowed to adapt. All procedures were approved by the Animal Care Committee at the University of Washington.

Experimental procedure. Animals were housed singly on Day 0. On Days 1-6 the *ob/ob* males were either injected with leptin (2 μg/g body weight, i.p.; n=5) and *ad lib*-fed or they were injected with an equivalent volume of the physiological saline vehicle and pair-fed (n=5). For pair-feeding, animals were fed daily an amount of food equivalent to that eaten by the leptin-treated mice. Pair-feeding was done as a control to ensure that any effects of leptin were not solely due to indirect effects of decreased food intake. Lean control males were injected daily with saline vehicle and fed *ad lib*. All injections were given just before lights off. Food intake and body weights were measured daily. Animals were sacrificed on the afternoon of Day 7 (1230-1500 h). For sacrifice, animals were asphyxiated with CO_2, blood was taken via heart puncture, and animals were decapitated. Brains were rapidly removed, frozen on dry ice, and stored at -80 C. Blood was allowed to clot at 4 C for at least 1 h and spun at 1500 x g for 15 min, and serum was stored at -20 C. Testes

were removed and weighed. Recombinant full-length human leptin was produced in *Saccharomyces cerevisiae*, purified by analytical HPLC, and quantified by mass spectroscopy.

In situ hybridization. Twenty-micron sections through the hypothalamus were cut on a cryostat, thaw-mounted onto Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA) and stored at -80 C. The plasmid vector containing a cDNA to mouse pituitary POMC mRNA was kindly provided by Dr. Michael Uhler. A 925-basepair HindIII-EcoRI fragment was subcloned into pSp64 (Promega Biotec, Madison, WI). An antisense POMC cRNA was synthesized with the Ambion MAXIscript kit and UTP, of which 7% was ^{35}S-labeled, and purified on a G25 spin column (Boehringer Mannheim, Indianapolis, IN). *In situ* hybridization was performed as described previously (28). Briefly, tissue was fixed and delipidated. The riboprobe was diluted in hybridization buffer to a final concentration of 0.2 μg/(ml x kb), applied to the tissue sections, covered with silanized coverslips and left to hybridize overnight at 55 C. Sections were then RNase treated, washed in SSC (to a stringency of 0.1x SSC at 60 C), and dehydrated through alcohols containing ammonium acetate. Slides were then dipped in NTB2 emulsion (Eastman, Kodak, Rochester, NY; diluted 1:1 with ammonium acetate) and stored at 4 C. Slides were then developed and counterstained with cresyl violet.

Image analysis. A mouse brain atlas (29) was used as a guide to match the coronal brain sections across animals. Four animals per group were analyzed. Sections were divided into 5 approximately equally sized contiguous areas; the retrochiasmatic area (RCh), the rostral arcuate [from enlargement of the third ventricle (3v) until the 3v completely descended], medial arcuate 1 (3v low, median eminence forms), medial arcuate 2 (full median eminence until the 3v ascends), and caudal arcuate (until the 3v splits). Slides were analyzed blindly with an automated image processing system, which determines the number of silver grains per cell, using a grain-counting program as previously described (30). The image processing system consisted of a PixelGrabber video acquisition board (Perceptics Corp., Knoxville, TN) attached to a Macintosh IIfx computer. Video images were obtained with a Dage model 65 camera (Dage-MTI, Michigan City, IN) attached to a Zeiss Axioskop (Zeiss, New York, NY).

Radioimmunoassay. Corticosterone was measured with an RIA kit (ICN, Costa Mesa, CA). Testosterone was measured using reagents supplied by the World Health Organization.

Statistical analysis. Data are presented as mean±SEM. Testicular weights and serum corticosterone and testosterone levels were analyzed with ANOVA followed by Fisher's PLSD test. Food intake was analyzed with a t-test. Cell count and grains/cell data were analyzed with 2-way ANOVA with repeated measures followed by Fisher's PLSD test.

Results

Grains per cell and cell number. POMC mRNA-containing cells were easily identified as dense clusters of silver grains throughout the RCh and ARC, and visual inspection of matched sections from representative animals illustrates that grain density differed among the three groups (Fig. 1). Quantitative analysis of the grain counts reflecting POMC mRNA revealed that POMC gene expression was dramatically reduced throughout the RCh and ARC in vehicle-treated *ob/ob* mice relative to that of lean controls ($p<0.0001$; Fig. 2). In addition, POMC mRNA levels were significantly increased in *ob/ob* animals receiving leptin compared to those receiving the vehicle alone ($p<0.0001$). A comparison of the total number of detectable POMC neurons in the arcuate revealed that in the rostral arcuate, leptin appeared to increase the number of cells that could be identified as expressing POMC mRNA (*ob/ob* control 50.5±1.6 cells/section; *ob/ob* leptin 73.6±6.9 cells/section; lean control 83.5±6.7 cells/section; $p<0.01$) whereas in the RCh and more caudal ARC—despite clear evidence for *regulation* of POMC mRNA by leptin— there did not appear to be any differences among the groups in actual cell counts (data not shown). This observation suggests that there is a unique population of neurons in the rostral ARC whose expression of the POMC gene is absolutely dependent upon the presence of leptin, without which there is virtually no (detectable) POMC message. Whether these particular POMC

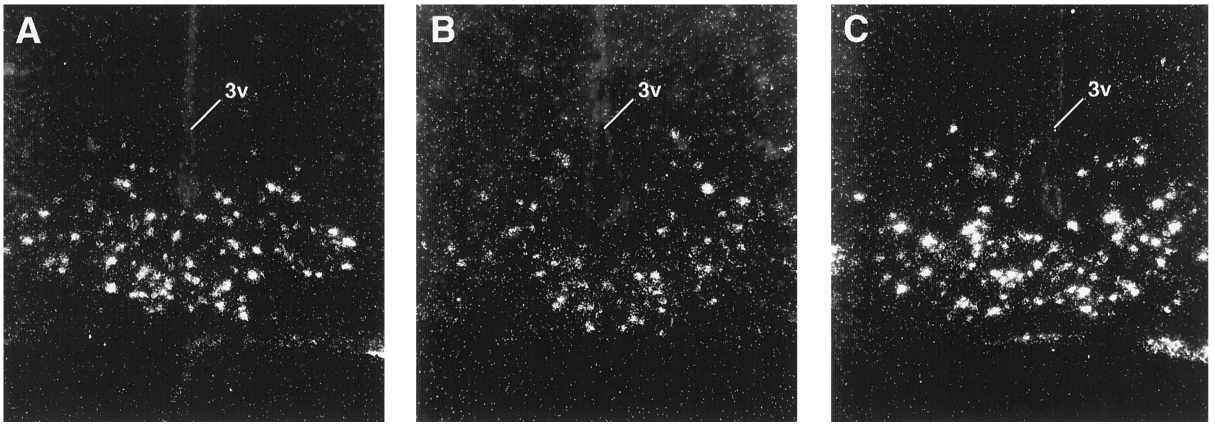

FIG. 1. Photomicrographs of POMC mRNA-containing cells in the rostral arcuate of A) wild-type lean control mice (vehicle-treated), B) *ob/ob* control mice (vehicle-treated and pair-fed), and C) *ob/ob* leptin-treated mice. Clusters of silver grains indicate the presence of cells containing POMC mRNA. Leptin significantly increased the number of grains/cell in the *ob/ob* mice to a level that was higher than that in the *ob/ob* controls ($p<0.0001$) and comparable to that in the lean control mice.

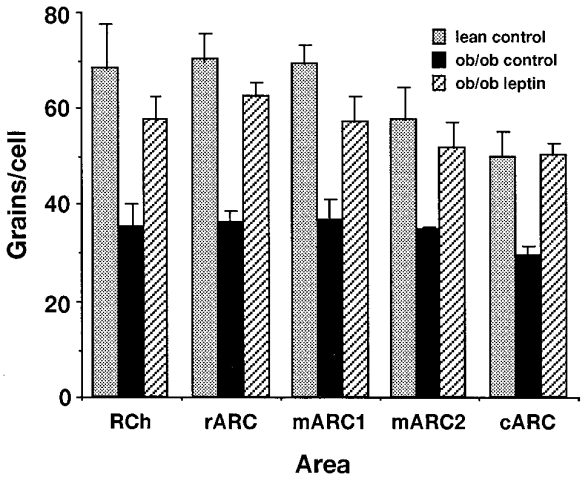

FIG. 2. POMC gene expression was reduced in the *ob/ob* control animals relative to the lean control males ($p<0.0001$) across the retrochiasmatic (RCh) area and arcuate nucleus including the rostral arcuate (rARC), the medial arcuate 1 and 2 (mARC1 and mARC2), and the caudal arcuate (cARC). Levels of POMC mRNA in cells throughout the RCh and ARC of the hypothalamus ($p<0.0001$) in the leptin-treated *ob/ob* mice were not significantly different from the lean control males.

neurons are those that express the leptin receptor or are simply unique by virtue of their synaptic input cannot be determined from this work; however, it does suggest that although leptin would appear to influence POMC gene expression in neurons throughout the arcuate, they are clearly not all of the same phenotype.

Food intake. As previously recognized, leptin treatment in this experiment resulted in a significant decrease in food intake in the *ob/ob* animals (leptin-treated *ob/ob* food intake Day 0=6.34±0.26 g vs Day 6=2.64±0.19 g; $p<0.0005$). Serum levels of corticosterone tended to be elevated in the *ob/ob* animals, and treatment with leptin resulted in their decline, although these differences did not reach statistical significance ($p=0.17$; Table 1). Testicular weights and serum levels of testosterone were low in the *ob/ob* mice, and treatment with leptin produced significant increases in these indices of reproductive function ($p<0.05$; Table 1).

Discussion

These results demonstrate that POMC gene expression in the hypothalamus is regulated by leptin. This suggests that one or more of the melanocortins, which are products of the POMC gene, are involved in mediating the effects of leptin on the brain. There are several different melanocortin receptor subtypes, including the MC4-R (23). Recent analyses of obese mice bearing either a targeted ablation of the MC4-R gene or the *agouti* mutation, in which the ectopically produced agouti protein blocks the MC4-R, suggests that the MC4-R is part of a hypothalamic circuit that inhibits feeding (20, 21). The phenotype of the cells expressing the MC4-R are unknown; however, likely candidates include NPY- and galanin-expressing cells in the hypothalamus, both of which have been implicated in the control of body weight and reproduction and could function either independently or as part of a network that regulates these complex physiological processes (10).

Since POMC neurons in the hypothalamus express leptin receptor, the most parsimonious interpretation of the present data is that leptin acts through its receptor directly on POMC cells, activating an intracelluar signaling pathway that regulates the POMC gene (22). However, leptin has target cells in many other regions of the brain as well as other organs of the body, which could indirectly influence the expression of POMC in the hypothalamus (11, 24). Indeed, animals that are chronically deficient in leptin (*ob/ob* mice) have profoundly abnormal circulating levels of testosterone (present data) and glucocorticoids (13), which are corrected when leptin is provided exogenously. These steroid hormones also have target cells in the hypothalamus (25), and since testosterone increases and glucocorticoids inhibit POMC gene expression (26, 27), it is conceivable that the leptin-dependent increase in circulating testosterone and reduction in corticosterone combine to induce the expression of POMC. Therefore, although POMC gene expression appears to be regulated directly by leptin, it may also be influenced by other hormones whose secretion is altered by leptin.

These observations add POMC gene products to the list of neuropeptides directly implicated in transducing the effects of leptin on the brain and suggest a unifying hypothesis for the role of melanocortins in the neuroendocrine regulation of both feeding and reproduction. In well-nourished animals, a sustained, high plasma level of leptin maintains POMC gene expression. Under these circumstances, one or more products of POMC acts on unidentified target neurons, which ultimately diminishes food-seeking behavior and supports the neuroendocrine reproductive axis. On the other hand, in poorly-nourished animals, chronically depressed levels of leptin and the consequent decline in POMC expression results in a disinhibition of feeding behavior and a reduction in the support for the neuroendocrine mechanisms driving gonadotropin secretion. Additional studies will be required to identify the POMC products involved in this process;

TABLE 1. Effect of leptin on testicular weight, testosterone, and corticosterone.

	Testicular weights (mg)	Testosterone (ng/ml)	Corticosterone (ng/ml)
lean control	193±1[a]	0.63±0.29	89±44
ob/ob control	138±1	0.04±0.04	241±62
ob/ob leptin	172±1[b]	1.03±0.17[c]	140±56

[a] $p<0.01$ compared to *ob/ob* control
[b] $p<0.05$ compared to *ob/ob* control
[c] $p<0.005$ compared to *ob/ob* control

however, one candidate is α-MSH, which has been shown both to inhibit food intake and to stimulate reproductive function (19-21).

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