

Central Leptin Gene Therapy Blocks Ovariectomy-Induced Adiposity

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Abstract

TORTO, RITA, STÉPHANE BOGHOSSIAN, MICHAEL G. DUBE, PUSHPA S. KALRA, AND SATYA P. KALRA. Central leptin gene therapy blocks ovariectomy-induced adiposity. *Obesity*. 2006;14:1312–1319.

Objective: In this study, we tested the hypothesis that insufficiency of leptin restraint in the hypothalamus is responsible for promoting weight gain and adiposity after ovariectomy (ovx). Whether increasing leptin transgene expression can overcome the diminution in leptin restraint was evaluated in ovx rats.

Research Methods and Procedures: Enhanced leptin or green fluorescent protein (GFP; control) transgene expression was induced by a single intracerebroventricular injection of recombinant adeno-associated viral vector encoding either leptin gene (*rAAV-lep*) or GFP gene (*rAAV-GFP*; control) in acutely and chronically ovx rats. Body weight and food intake responses were monitored weekly. White adipose tissue (WAT) mass and serum levels of WAT-derived hormones, leptin, and adiponectin were analyzed at termination of the experiments.

Results and Discussion: An increase in leptin transgene expression in the hypothalamus initiated soon after ovx blocked hyperphagia and body weight gain and markedly suppressed WAT mass and adipokines, leptin, and adiponectin. Similar suppression of weight gain and adiposity and serum leptin and adiponectin levels after intracerebroventricular *rAAV-lep* injection in chronically ovx rats were observed concomitant with unchanged daily food intake. These findings are consistent with the hypothesis that in the absence of ovarian steroids, the existent insufficiency of leptin restraint at the hypothalamic level can be overcome

with ectopic leptin expression, thereby reinstating central control on weight and adiposity.

Key words: leptin insufficiency, hypothalamus, adiposity, ovariectomy, gene therapy

Introduction

It is well-established that ovariectomy (ovx)¹ promotes hyperphagia and weight gain in rodents and humans (1,2). An analysis of the time course of these two responses showed that although hyperphagia is transient, body weight (BW) gain is longer lasting (3–9). Because estrogen replacement decreases food intake (FI) and restores weight in ovx rats (3–15), it has been suggested that normally estrogen participates in modulation of energy homeostasis by direct action on central appetite and energy-regulating pathways (16–20) and also by regulating the secretion of those hormonal signals from the periphery, e.g., leptin, that exert a feedback control on these central effector pathways (2,6,9,11–14,18,21).

Leptin, produced primarily by white adipose tissue (WAT) and hypothalamus (17–20,22), is essential for hypothalamic integration of energy homeostasis (17–20, 23,24). Leptin exerts a tonic restraint on weight by engaging leptin receptor (Ob-Rb)-expressing targets in the hypothalamus (17–20,25). In the absence of leptin restraint in the hypothalamus as seen in leptin and leptin receptor mutant rodents and humans (17–20,26–28), relentless hyperphagia accompanied by a steady increase in weight leading to gross obesity is the norm. Leptin replacement, either peripherally or centrally in leptin-deficient subjects (17,26,29,30), or instillation of leptin receptor selectively in the hypothalamus of Ob-Rb mutant rats normalizes FI and BW (28).

By employing gene transfer technology with the aid of central administration of a non-pathogenic and non-immunogenic recombinant adeno-associated viral vector encod-

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¹ Nonstandard abbreviations: ovx, ovariectomy; BW, body weight; FI, food intake; WAT, white adipose tissue; BAT, brown adipose tissue; GFP, green fluorescent protein; PF, pair fed; icv, intracerebroventricular(ly); UCP-1, uncoupling protein-1; RT, reverse transcription; PCR, polymerase chain reaction.

ing the leptin gene (*rAAV-lep*), we observed blockade of the gradual age-related weight gain in rats and mice (23,24,31–38). Enhancement of leptin availability locally in the hypothalamus by central administration of *rAAV-lep* also suppressed the rapid weight gain and obesity induced by consumption of a high-fat diet (39). A common consequence of increased leptin transgene expression selectively in the hypothalamus was a dramatic depletion of body fat and enhanced thermogenic energy expenditure mediated by activation of brown adipose tissue (BAT), concomitant with or without a restraint on FI (23,24,30–38).

To ascertain the role of leptin in the ovx-induced weight gain, numerous investigations have evaluated the effects of ovx on circulating leptin levels (3,5,7,9,11,15). We observed that ovx led to a rapid decrease in episodic leptin secretion that temporally correlated with hyperphagia and weight gain (6). On the other hand, long-term increases in BW and fat mass in association with hyperleptinemia have also been seen in ovx rats (1,3,4,7,12,13). Because diminution in central leptin restraint results in increased FI and weight gain, we speculated that a deficiency in leptin restraint in the hypothalamus after ovx may underlie hyperphagia and increased weight gain and fat deposition. To validate this possibility, we evaluated the acute and long-term effects of increased leptin transgene expression in the hypothalamus with the aid of central leptin gene transfer on hyperphagia, weight gain, and adiposity in ovx rats.

Research Methods and Procedures

Animals

Adult female Sprague-Dawley rats weighing 230 to 250 grams were purchased from Harlan (Indianapolis, IN). Rats were housed individually in a temperature (22 °C to 25 °C)- and light/dark (lights on 5 AM to 7 PM)-controlled, specific pathogen-free room. Standard rat chow and water were available ad libitum. The animal use protocol was approved by the Institutional Animal Care and Use Committee.

Construction and Packaging of rAAV Vectors

To enhance leptin transgene expression in the hypothalamus, a recombinant non-immunogenic, non-pathogenic, and replicative-deficient rAAV encoding either rat leptin or green fluorescent protein (GFP) gene was packaged, purified, concentrated, and titered in the vector core laboratory at the University of Florida as described and used in our previous studies (23,24). These vectors were employed for the following two studies to evaluate the effects of enhanced leptin expression on BW, FI, and other parameters of adiposity in response to bilateral ovx.

Experiment 1: Evaluate the Effects of Central Leptin Gene Therapy on the Effects of OVX on BW and FI

Rats anesthetized with ketamine-xylazine (100 mg/kg ketamine + 15 mg/kg xylazine BW) were bilaterally ovari-

ectomized and then stereotaxically implanted with a permanent stainless steel cannula in the third cerebroventricle as described earlier (23,24,32,39). The control groups monitored in parallel were: ovary intact, unoperated ($n = 6$) and ovariectomized ($n = 6$) and pair fed (PF; $n = 6$) to the amount of food consumed by ovx rats injected with *rAAV-lep*. After 1 week of recovery, rats were weight matched and injected intracerebroventricularly (icv) with either *rAAV-lep* (5 μ L, 4.6×10^{13} infectious particles/mL, $n = 7$) or *rAAV-GFP* (5 μ L, 4.29×10^{12} infectious particles/mL, $n = 6$). BW and FI were monitored weekly for 10 weeks. At the end of Week 10, rats were sacrificed by decapitation. Blood samples were collected from trunk, and serum was kept frozen at -20 °C for hormone analyses. Abdominal WAT was dissected and weighed. Brains were removed and hypothalamus excised and stored in RNAlater (Ambion, Austin, TX) for analyses of leptin mRNA as described (23,24,31,32). BAT was dissected and stored in RNAlater for analysis of uncoupling protein-1 (UCP-1) mRNA by dot-blot analysis (23,24,31,32).

Experiment 2: Effects of Central Leptin Gene Therapy in Obese ovx Rats

Rats were ovariectomized and allowed to gain weight for 6 weeks. Thereafter, a permanent stainless steel cannula in the third cerebroventricle was implanted as in Experiment 1. Two weeks later (i.e., 8 weeks post-ovx) rats were weight matched and received either *rAAV-lep* ($n = 8$) or *rAAV-GFP* ($n = 7$) as described above. An additional control group consisted of ovariectomized untreated rats ($n = 7$). Rats were sacrificed by decapitation 14 weeks later. Blood samples, hypothalamus, and BAT were collected and processed as described in Experiment 1.

Analyses

Reverse Transcription (RT)-Polymerase Chain Reaction (PCR) for Leptin Gene Expression. Leptin gene expression was analyzed by RT-PCR to confirm leptin mRNA expression in the hypothalamus of rats injected with rAAV-leptin. Total RNA was extracted with the RNeasy kit (QIAGEN Inc., Valencia, CA). First strand cDNA was obtained from RT of 1 μ g of RNA using an RT system (Promega, Madison, WI) according to the manufacturer's instructions. Primers for rat leptin were sense, 5' TGACACCAAAC-CCTCATCA 3'; and antisense, 5' ATCCAGGCTCTCGGC-TTCT 3'. Cyclophilin was used as endogenous control. A 199-base pair fragment was generated with primers: sense, 5' ATGTGGTACGGAAGGTGGAG 3'; and antisense, 5' TGGCTACCTTCGTCTGTGTG 3'. The designed primers were purchased from MWG (High Point, NC). Leptin mRNA expression was analyzed by RT-PCR as previously described (23,24,31,32).

Dot-Blot Analyses for UCP-1. Total RNA was extracted from BAT using an RNA isolation kit (STAT-60; Teltest

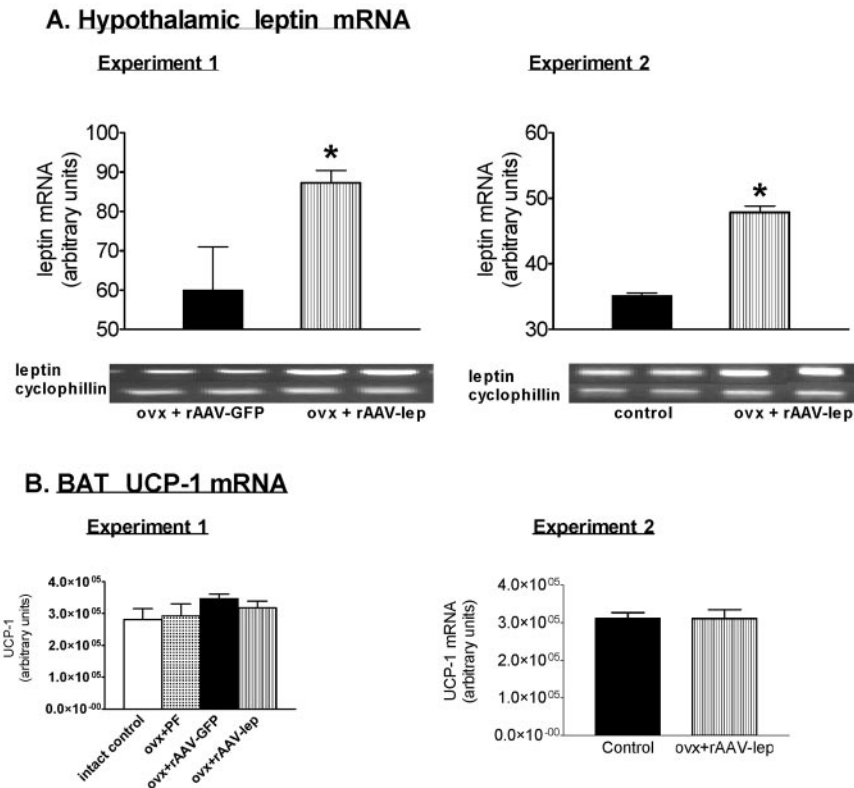


Figure 1: Hypothalamic leptin mRNA (A) and BAT UCP1 mRNA (B) expression in control (*rAAV-GFP*) and *rAAV-lep*-injected ovx rats sacrificed at 10 (Experiment 1) or 14 (Experiment 2) weeks post-injection. * $p < 0.05$

Inc., Friendswood, TX) and analyzed for UCP-1 mRNA expression by dot-blot hybridization as previously described (23,24,31,32). The UCP-1 probe was made from UCP-1 cDNA (provided by Dr. Leslie Kozak, Jackson Laboratory, Bar Harbor, ME) and prime labeled using a Prime-A-Gene kit (Promega) and purified through a Nick column (Amersham Pharmacia Biotech AB, Uppsala, Sweden).

Radioimmunoassays. Serum leptin and adiponectin levels were assayed using the rat leptin and adiponectin radioimmunoassay kits from Linco Research, Inc. (St Louis, MO) according to the manufacturer's instructions (1,23,24,31). The assay sensitivities and range of detection were leptin, 0.5 to 50 ng/mL; insulin, 0.1 to 10.0 ng/mL; and adiponectin, 1 to 100 ng/mL.

Statistical Analyses. Weekly BW and FI were analyzed using a two-way repeated measures ANOVA with time and treatment as variables. Hypothalamic leptin mRNA, UCP-1 mRNA, plasma leptin, and adiponectin levels and WAT tissue mass were compared by one-way ANOVA and post hoc analyses with Bonferroni's multiple comparison test or Student's *t* test, as appropriate. Significance was set at $p < 0.05$ for all analyses.

Results

Effects of Hypothalamic Leptin Transgene Expression on FI, BW, and UCP-1 mRNA in ovx Rats

In agreement with previous investigations (22–24, 32,35,36), low expression of leptin mRNA was detected in the hypothalami of control *rAAV-GFP*-treated rats, and *rAAV-lep* injection increased leptin mRNA expression significantly ($p < 0.05$) at Weeks 10 (Experiment 1) and 14 (Experiment 2) post-injection (Figure 1A). In contrast, *rAAV-lep* failed to affect BAT UCP1 mRNA expression in the two experiments (Figure 1B).

The effects of increased hypothalamic transgene expression on BW and FI in acutely ovx rats are shown in Figure 2. Daily food consumption increased rapidly after ovx and was significantly higher at Week 1 ($p < 0.05$ vs. intact controls). In ovx rats receiving *rAAV-GFP*, FI increased further until Week 3. Thereafter, energy intake gradually decreased to the range found in intact untreated control group at Week 10. In contrast, *rAAV-lep* injection arrested the ovx-induced rise in FI, and the amount consumed by these rats remained stable and at a significantly lower level than that in *rAAV-GFP* controls for up to 8 weeks post-

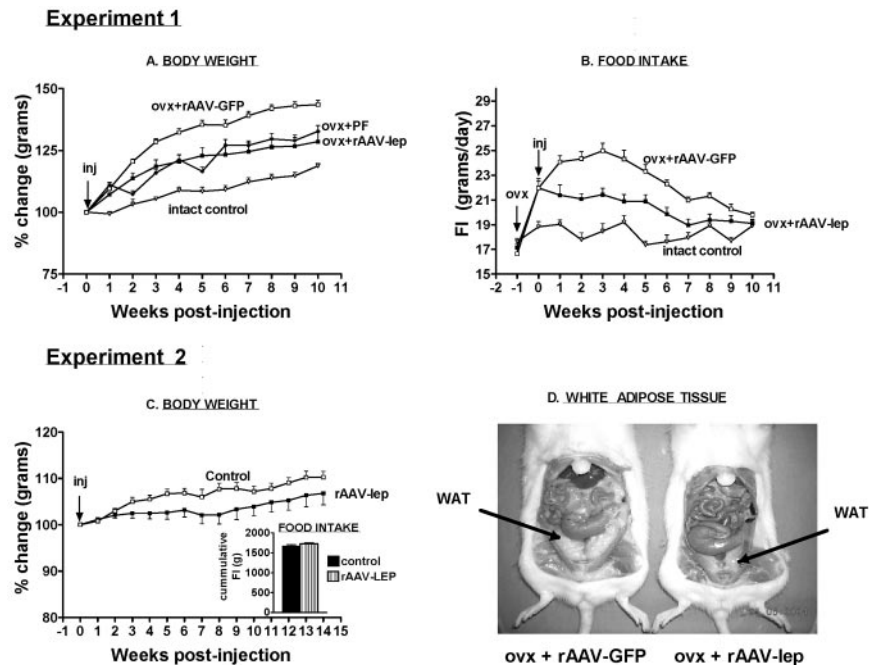


Figure 2: Experiment 1: the effects of icv *rAAV-lep* injection immediately after ovx on BW (A) and FI (B). Experiment 2: the effects of icv *rAAV-lep* injection 8 weeks after ovx on BW (C) and FI (C, inset) monitored for 14 weeks post-injection. Control group represents data combined from untreated and *rAAV-GFP*-injected rats. Reduction in WAT is shown in panel D in a representative *rAAV-lep*- vs. *rAAV-GFP*-treated rat.

injection. However, by Week 10, FI of control *rAAV-GFP* and *rAAV-lep* groups of rats was similar to that of the intact untreated rats.

The effects of ovx on BW were different in both control *rAAV-GFP* and *rAAV-lep* experimental groups. In contrast to the transient increase in energy intake, BW continued to increase gradually to 43.5% above the pre-ovx levels at 10 weeks post-ovx in control *rAAV-GFP*-treated rats ($p < 0.05$). On the other hand, enhanced hypothalamic leptin transgene expression promptly blocked this ovx-induced accelerated weight gain. Suppression of weight gain in *rAAV-lep*-treated rats was evident at Week 2 post-injection ($p < 0.05$), and this response was maintained through the remaining 8-week period of observation. The magnitude of weight suppression in *rAAV-lep*-treated rats and in ovx, PF rats was similar.

As shown in Figures 2 and 3, increased weight gain in *rAAV-GFP*-treated control ovx rats was accompanied by increased abdominal WAT mass as compared with that found in intact rats ($p < 0.05$). Likewise, plasma leptin levels were markedly increased in these rats ($p < 0.05$, Figure 3). Plasma adiponectin, a product of adipose tissue, was unaffected by ovx and PF (20,30,37,40). However, *rAAV-lep* treatment markedly suppressed plasma leptin and adiponectin levels as compared with those in the control *rAAV-GFP* group of rats.

The effects of enhanced leptin transgene expression in the hypothalamus (Figure 1) on BW and FI responses in chron-

ically ovx rats in Experiment 2 were different (Figure 2). Because BW and FI intake responses in untreated ovx control and *rAAV-GFP*-treated control ovx rats were similar, the results of these two control groups were combined for comparison with the *rAAV-lep* experimental group. As in acutely ovx rats in Experiment 1, *rAAV-lep* injection suppressed BW gain in chronically ovx obese rats ($p < 0.05$, Figure 2). Although BW continued to increase gradually in a time-related manner in control rats, *rAAV-lep* injection arrested this slow rate of weight gain. From Week 2 post-injection onward, the BW of these rats remained stable and below the control range for the duration of the experiment ($p < 0.05$). However, FI was unchanged after *rAAV-lep* injection in these rats (Figure 2C, inset). As in Experiment 1, the suppression of BW was associated with significantly diminished WAT mass and the adipokines, leptin and adiponectin ($p < 0.05$, Figure 3).

Discussion

Previous neuroanatomical mapping studies and quantitation of leptin mRNA expression after *rAAV-lep* injection demonstrated enhanced leptin transgene expression in those hypothalamic sites that express the biologically relevant Ob-Rb and participate in integration of energy balance on a daily basis (18,23–25,31,32,34–37). It was also evident that the transduced leptin protein in these target sites alone, without leakage to either cerebrospinal fluid or to the pe-

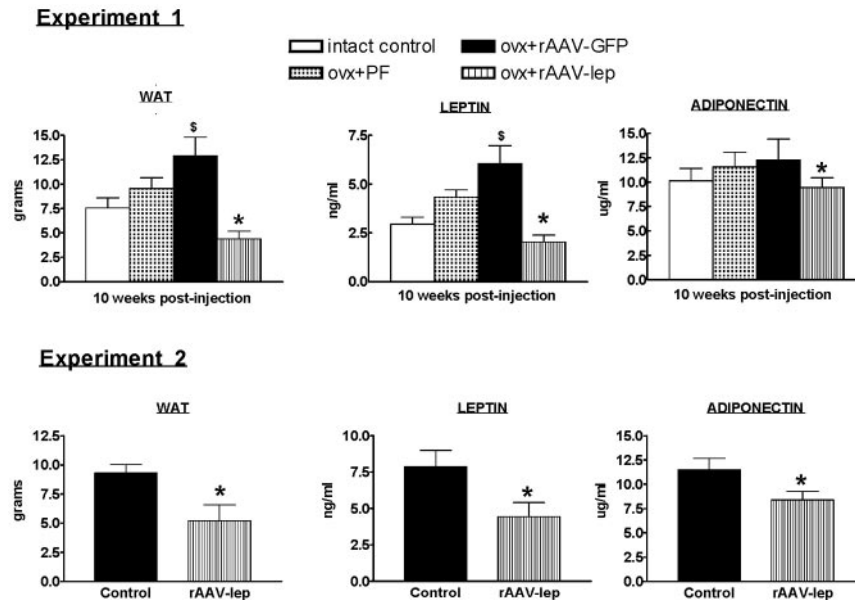


Figure 3: Effects of icv *rAAV-lep* injection on abdominal WAT mass and serum leptin and adiponectin levels at 10 (Experiment 1) and 14 (Experiment 2) weeks post-injection. \$ $p < 0.05$ vs. all groups; * $p < 0.05$ vs. *rAAV-GFP* or control as applicable.

riphery, was sufficient to exert long-term restraint on the age-related gradual weight gain in gonad-intact rodents (23,24,30,34–37). The results of the current study demonstrate that increase in leptin mRNA expression in the hypothalamus initiated immediately after ovx is, likewise, effective in blocking the transient hyperphagia and the attendant weight gain for the entire period of observation. In addition, we found that a similar increase in the local supply of leptin in the hypothalamus induced several weeks post-ovx, when rats were already obese, promptly decelerated the normally occurring gradual time-related weight gain even in the presence of unchanged daily FI. A similar separation of the effects on BW and FI were seen after either icv injection of extremely low doses of *rAAV-lep* or microinjection of *rAAV-lep* centrally into the brain stem (23,24,31,35,36,41). Also, a curb on weight gain and adiposity without decreases in FI has been observed in response to infusion of leptin for long periods either centrally or peripherally (5,9,18, 29,33,42,43). Seemingly, decreased BW after *rAAV-lep* injection in acutely ovx rats is due to retardation of fat deposition as evidenced by diminished WAT mass and the adipocyte secretory products, leptin and adiponectin (23,24,34,37,40). The *rAAV-lep* treatment similarly decreased the fat mass and plasma leptin and adiponectin concentrations without affecting lean body mass in intact rodents (23,24,30,32,35–37). The similar diminution in adiposity seen in chronically ovx rats indicates that *rAAV-lep* treatment either slowed the rate of fat deposition with time or increased the rate of fat depletion.

This suppression of BW attributable to a selective reduction in fat mass in the presence of normal daily FI in

rAAV-lep-treated ovx rats may result from increased energy expenditure, lipolysis, and adipocyte apoptosis (5,11,29, 33,34,37,42,44,45). In gonad-intact rats, central leptin injection and leptin transgene expression stimulate BAT thermogenic energy expenditure concomitant with both diminished and normal FI (5,11,23,24,28,31,32,42,43). In contrast, we observed that *rAAV-lep* failed to increase BAT UCP1 mRNA expression, a marker of non-shivering thermogenesis (23,24,33,34,37). These unexpected observations suggest that increased BAT-mediated thermogenesis in response to central leptin may require estrogen milieu, an observation that warrants additional investigation. Similarly, additional studies will be required to ascertain whether enhanced central leptin gene expression increases the rate of lipolysis leading to fat depletion, as also seen in intact rodents after peripheral and central infusion of leptin (5,11,42–44) or whether loss of fat mass in *rAAV-lep*-treated rats is due to increased apoptosis of adipocytes, as seen after central administration of leptin (34,37,45).

The current findings taken together with previous demonstrations that central leptin gene therapy suppresses weight gain and adiposity in intact rodents either consuming regular rat chow or a high-fat diet (23,24,31,32,35–37,39) are consistent with the inference that, similar to that seen in gonad-intact rodents, insufficiency of central leptin restraint is also responsible for increased adiposity after ovx and that it can be prevented by enhanced leptin transgene expression in the hypothalamus (21,33,46).

Analogous to the impact of central leptin transgene expression, estrogen administered by either peripheral or central routes reinstates weight homeostasis in ovx rats (1,9–

11,15,47). In fact, diminution in the release of the orexigenic neuropeptide, neuropeptide Y, in the paraventricular nucleus is apparently responsible, in part, for the estrogen-induced decrease in energy intake and weight suppression (16,18,48). In addition, activation by estrogen of hypothalamic anorexigenic melanocortin signaling reportedly participates in reinstatement of weight homeostasis (18–20). *rAAV-lep* treatment also diminishes orexigenic neuropeptide Y and augments anorexigenic melanocortin signaling in the hypothalamus (23,24,31,32,34,37). Thus, it is possible that the beneficial effects of estrogen on weight control may be mediated by the estrogen-induced dynamic modifications in central leptin feedback action. One can envision that estrogen may act synergistically with leptin at the central effector targets to enhance leptin restraint on weight gain. At the neuroanatomical level, it has been shown that neurons in the arcuate nucleus of the basal hypothalamus involved in energy homeostasis, especially the orexigenic neuropeptide Y and anorexigenic melanocortin-stimulating hormone-expressing subpopulations, coexpress leptin and estrogen receptors (18,19,49,50). Thus, it is likely that an interplay of leptin and estrogen at the level of hypothalamic energy regulating pathways may be important in sustaining weight homeostasis in gonad intact rodents. The observation that mice lacking estrogen receptor α are obese (47) underscores the possibility that estrogen insufficiency in this leptin-estrogen interplay disrupts weight homeostasis. Consequently, we suggest that in the absence of estrogen, as in ovx rats, a higher level of hypothalamic leptin restraint on energy balance imposed by increased hypothalamic leptin transgene expression is able to reinstate weight homeostasis in a manner elicited by the synergistic interplay of estrogen and leptin in intact rats. Additional investigations are underway to affirm the notion that leptin-estrogen interplay at the level of hypothalamic effector pathways plays an important role in weight homeostasis.

Additionally, the inability of peripheral hyperleptinemia in chronically ovx rats (3–9), but not of increased leptin locally in the hypothalamus of *rAAV-lep*-treated rodents, requires explanation. Leptin levels in the cerebrospinal fluid do not rise in conjunction with hyperleptinemia, and hypothalamic Ob-Rb mRNA levels are compromised after ovx (7,9,14,51,52). These observations can be viewed as suggestive of the existence of insufficiency of central leptin restraint that, as our studies suggest, is reversible with increased central supply of leptin. However, several lines of evidence also advocate that the anorectic and weight-reducing effects of estrogen manifest independent of leptin (5,8,11,14,15,47). Thus, the issue of whether and how leptin normally participates in the weight-reducing effects of estrogen remain to be clarified. Nevertheless, the current outcome of the leptin gene transfer paradigm implies that in the absence of ovarian steroids, the insufficiency in hypothalamic leptin restraint may be overcome by ectopic leptin

expression in the hypothalamus and thereby restore weight homeostasis for extended periods.

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