

Long-Term Suppression of Weight Gain, Adiposity, and Serum Insulin by Central Leptin Gene Therapy in Prepubertal Rats: Effects on Serum Ghrelin and Appetite-Regulating Genes

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ABSTRACT

Intracerebroventricular administration of recombinant adeno-associated virus (rAAV) encoding the rat leptin gene (rAAV-lep) to 24-d-old female and male rats suppressed postpubertal weight gain for extended periods by decreasing food consumption and adiposity, as reflected by lowered serum leptin, insulin, and FFA. Serum ghrelin levels were increased in young but not older rats. Central rAAV-lep therapy also increased energy expenditure through nonshivering thermogenesis in younger rats as shown by expression of uncoupling protein mRNA in brown adipose tissue. The sustained decrease in appetite seemingly resulted from attenuation of appetite-stimulating neuropeptide Y and enhancement of appetite-inhibiting melanocortin signalings in the hypothalamus. Neither the onset of pubertal sexual maturation nor reproductive cyclicality in adult female rats was affected by the sustained reduction in energy consumption and weight gain. These findings demonstrate that central leptin gene therapy in prepubertal rats is a novel therapy to control postpubertal weight gain, adiposity, and hyperinsulinemia for extended periods. (*Pediatr Res* 52: 189–198, 2002)

Abbreviations

rAAV, adeno-associated virus
BAT, brown adipose tissue
BW, body weight
ARN, appetite-regulating network
lep, leptin
icv, intracerebroventricular
NPY, neuropeptide Y
FI, food intake
UCP1, uncoupling protein 1
POMC, proopiomelanocortin
AGRP, *agouti*-related peptide
 α -MSH, α -melanocortin stimulating hormone
GFP, green fluorescent protein
CSF, cerebrospinal fluid
ARC, arcuate nucleus
PF, pair-feeding
PVN, paraventricular nucleus

Considerable attention has focused recently on the role of leptin in the hypothalamic integration of energy homeostasis (1, 2). Although produced primarily by white adipose tissue and released into the general circulation, other tissues, including the hypothalamus, have been reported to produce leptin for paracrine/autocrine action (3, 4). Experimental evidence shows that peripheral leptin is transported across the blood-brain barrier to the hypothalamus to regulate BW by dual action (1, 5). Leptin restrains food intake

by modulating the ARN (5) and, simultaneously, it enhances the nonshivering thermogenic energy expenditure by BAT (6, 7). However, resistance to peripheral leptin develops readily in rodents and humans with aging (8–10) and after consumption of calorie-rich diets (11–15), each culminating in increased fat deposition and obesity and a rise in circulating leptin levels in direct proportion to fat depots. Because leptin levels in CSF do not increase in proportion to the peripheral concentrations (16–18) and leptin administered centrally is effective despite elevated peripheral leptin in diet-induced obese rodents (19), defective leptin transport to central sites may be a causal factor in the loss of leptin control. Consequently, newer experimental approaches have recently been explored either to circumvent the impaired leptin transport or to overcome leptin insufficiency at hypothalamic sites (20).

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Gene transfer by rAAV vectors to correct deficiencies underlying CNS diseases has been extensively investigated in rodents (21–23). The feasibility of generating rAAV vectors at high titers for long-term transgene expression in nondividing cells without eliciting an immunogenic response is the distinct advantages of rAAV over other viral vectors (22, 23). We generated a rAAV vector encoding leptin cDNA (rAAV-lep) and evaluated its efficacy in leptin-deficient *ob/ob* mice and in normal adult rats (10, 15, 24, 25). Results demonstrated that a single icv injection of rAAV-lep increased leptin transgene expression in the hypothalamus, leading to a sustained reduction in weight gain and adiposity in rats consuming either regular rat chow or a high-fat diet. A drastic reduction in hyperleptinemia and hyperinsulinemia in conjunction with normoglycemia was noted in these rats. Reduction in energy consumption and/or increased thermogenic energy expenditure, as indicated by enhanced uncoupling protein-1 mRNA in the BAT (6, 7), accounted for the sustained loss of adiposity. An evaluation of the leptin transgene action on hypothalamic ARN showed a decrease in the appetite-stimulating NPY concurrent with an increase in inhibitory melanocortin signaling (24).

Obesity presents a worldwide health problem as it is a major cause of hypertension, stroke, non-insulin-dependent diabetes, and increased morbidity (26). A recent census showed a rise in pediatric obesity, with large numbers of overweight children and adolescents who are at risk to become overweight adults and face similar health risks (27). Available pharmacologic approaches are cumbersome, transiently effective, and require repeated treatments. Because a single administration of rAAV-lep in adult rats produced weight reduction and prevented obesity for 6 mo duration of the experiment (25), the primary aim of this undertaking was to ascertain whether central leptin gene therapy will be effective in maturing rats for an extended period. The first objective of this study was to assess the long-term efficacy of central rAAV-lep therapy in prepubertal rats on FI, weight gain, and adiposity during the pubertal and postpubertal periods. Because hyperinsulinemia is closely associated with obesity (1, 2, 28, 29), the second objective was to evaluate the long-term effects of central leptin overexpression on circulating insulin and glucose levels.

Our previous studies in adult rats showed that leptin transgene expression in the hypothalamus markedly increased thermogenic energy expenditure as indicated by increased BAT UCP1 mRNA expression (24, 25). Our third objective was to determine whether rAAV-lep therapy started prepubertally would sustain increased energy expenditure into adulthood. As leptin injections in adult rats down-regulate orexigenic NPY and AGRP and up-regulate the anorexigenic peptide α -MSH, derived from the *POMC* gene (1–3, 30), the fourth objective was to evaluate whether leptin overexpression in the hypothalamus would exert sustained effects on these two functionally opposing, appetite-regulating, peptidergic systems in the hypothalamus.

Ghrelin, a peptide produced in the stomach (31), has recently been shown to stimulate feeding by increasing NPY release in the hypothalamus (32–34). Ghrelin secretion is increased after fasting and this increment may play a role in stimulating

appetite (34). Our fifth objective was to analyze circulating ghrelin levels in rats displaying reduced food intake in response to hypothalamic leptin-overexpression.

Finally, a secondary objective of this study was to evaluate neuroendocrine effects of central rAAV-lep therapy in noninvasive manner so as not to compromise these five major objectives. There is evidence that leptin may be involved in the neuroendocrine regulation of sexual maturation and reproductive cycles (35, 36). Administration of leptin prepubertally was reported by some but not all investigators to advance the onset of puberty (35–37). A delay in sexual maturation in response to drastic caloric restriction prepubertally was prevented by leptin administration (37–40). Similarly, fasting and severe caloric restriction in adult rodents have been shown to interrupt estrous cycles (41, 42). The sixth objective of this investigation was to evaluate the impact of reduced voluntary caloric intake produced by prepubertal central leptin gene therapy on the onset of puberty and postpubertal estrous cycle patterns, the evaluation parameters of which are noninvasive.

MATERIALS AND METHODS

Experimental Animals

Twenty-one-day-old female and male Sprague Dawley rats (35–60 g) were purchased from Harlan Sprague Dawley (Indianapolis, IN, U.S.A.) and housed individually in a temperature- (22–25°C) and light-controlled (14 h light, 10 h dark), specific-pathogen-free environment. Animals were fed standard rat chow *ad libitum* (Teklad, Madison, WI, U.S.A.) and water was available at all times. The experimental protocols were approved by the Institutional Animal Care and Use Committee.

Construction and Packaging of rAAV Vectors

The rAAV-vectors used in this study were constructed at the University of Florida Gene Therapy Center. Briefly, the vector pTR-CBA-Ob *EcoRI* fragment of pCR-rOb (a gift of Dr. Roger H. Unger, Southwestern Medical School, Dallas, TX, U.S.A.) containing rat leptin cDNA was subcloned into rAAV vector plasmid pAAV β GENh after deletion of the *EcoRI* fragment carrying β -glucuronidase cDNA sequence. Vectors were packaged, purified, concentrated, and titered essentially as described previously (10, 24, 25, 43). The titer of rAAV-CBA-Ob vector, hereafter referred to as rAAV-lep, was 2.3×10^{13} physical particles/mL. The ratio of physical-to-infectious particles was <100 . The control vector rAAV-UF5, encoding the GFP (43), was similarly constructed. The titer of the rAAV-UF5 used was 3×10^{13} physical particles/mL.

Experimental Design

To evaluate the effects of icv injection of rAAV-lep on BW, FI and metabolic hormones in the blood, 22-d-old rats were anesthetized with an i.p. injection of ketamine/xylazine (ketamine 100 mg/kg BW + xylazine 15 mg/kg BW) and stereotaxically implanted with a permanent cannula in the third cerebroventricle, and CSF efflux served as an indicator of accuracy of cannula placement (24, 25).

Experiment 1: long-term study. Rats bearing icv cannula were divided into two weight-matched groups (eight to 10 rats per group) on d 24 and injected icv (3 μ L) with either rAAV-UF5 (control, 9×10^{10} particles) or rAAV-lep (6.9×10^{10} particles). Selection of these titers was based on our previous studies (24, 25). A third group of weight-matched rats consisting of unoperated, untreated rats ($n = 8$) was monitored in parallel. BW and 24-h FI were recorded before the injection and twice a week thereafter for 294 d. To determine the timing of sexual maturation in female rats, vaginal opening was assessed by daily observation from d 24 onward. After vaginal opening, vaginal cytology of each female was recorded (44–46) to determine the numbers of estrous cycle and cycle length during d 54–70, 120–146, 186–240, and 240–270.

Experiment 2: short-term studies. In the first experiment, female rats were divided into two weight-matched groups (seven to nine rats per group) and injected icv (3 μ L) with either rAAV-UF5 or rAAV-lep, as described in experiment 1. The timing of vaginal opening and estrous cycle length between d 54–70 was assessed as described in experiment 1. BW and 24-h FI were monitored twice a week for 89 d postinjection. Additionally, there were three control groups: 1) unoperated, unoperated rats ($n = 8$); 2) sham-operated rats ($n = 7$); and 3) unoperated, rats pair-fed to the amount consumed by rats receiving rAAV-lep ($n = 7$). In the second experiment, male rats were divided into two weight-matched groups (seven to 11 rats per group), and injected icv either with rAAV-UF5 or rAAV-lep as described in experiment 1. Control groups consisted of sham-operated animals ($n = 8$), unoperated, untreated rats ($n = 6$), and unoperated, untreated rats ($n = 7$) pair-fed to the amount consumed by rats receiving rAAV-lep treatment. BW and 24-h FI were monitored once a week for 111 d postinjection.

At the end of the experiments, rats were killed between 0900 and 1200 h. Brain and BAT tissues were dissected out and rapidly frozen at -80°C . Serum from trunk blood was collected and stored at -20°C until analyses.

Analyses

RT-PCR. To confirm hypothalamic leptin mRNA expression, hypothalami from rAAV-lep- and rAAV-UF5-injected and untreated rats ($n = 4$ per group) were dissected from the brain on d 294 postinjection (long-term study, experiment 1). Total RNA was extracted using RNA STAT 60 RNA isolation kit (Tel-Test Inc., Friendswood, TX, U.S.A.). First-strand cDNA was obtained by using an RNA PCR kit (Applied Biosystems, Foster City, CA, U.S.A.) and reverse-transcriptase PCR for leptin mRNA was conducted as previously described (24, 25). The relative values of mRNA levels were derived from comparison of the intensities of the target and simultaneously run internal controls (cyclophilin). All PCR products were run on a single gel to control for inter-gel variation.

Analyses of hormones and metabolic variables. Serum leptin and insulin levels were measured in duplicate using rat leptin and insulin RIA kits from Linco Research Inc. (St. Charles, MO, U.S.A.). The assay sensitivity for leptin was 0.5 ng/mL and the range of detection was between 0.5 and 50

ng/mL. The assay sensitivity for the insulin assay was 0.1 ng/mL and the range of detection was between 0.1 and 10 ng/mL. Serum glucose was measured using Trinder, a Sigma Chemical (St. Louis, MO, U.S.A.) diagnostic glucose reagent based on a quantitative, colorimetric, enzymatic reaction read at 505 nm. Serum FFA levels were measured using an ACS (acyl-CoA synthetase)-ACOD (acyl-CoA oxidase) method (NEFA C kit, Wako Chemicals USA, Richmond, VA, U.S.A.). Serum ghrelin levels were assayed in duplicate using a rat ghrelin RIA kit from Phoenix Pharmaceuticals (Mountain View, CA, U.S.A.). The assay sensitivity was 0.01 ng/mL and the range of detection was between 0.01 and 1.28 ng/mL.

Dot blot analysis for BAT UCP-1 mRNA. BAT UCP-1 mRNA was analyzed as described (24, 25).

In situ hybridization. Semiquantitative analyses of gene expression of *NPY*, *AGRP*, and *POMC* in the hypothalamic ARC of female rats ($n = 3$) in experiments 1 and 2 was conducted as previously described (10, 24). Twelve matched sections containing the ARC from each brain were analyzed. We estimated the relative OD (ROD), calculated as total target area multiplied by the integrated OD for *AGRP*, *NPY*, and *POMC*, from autoradiograms with the MCID image analysis system (Imaging Research, St. Catharines, Ontario, Canada). The background OD in an area adjacent to the ARC was subtracted from the target OD. The ROD of 12 sections in the same brain were averaged and expressed relative to the average ROD from the control group.

Statistical analyses. BW and FI data were analyzed using two-way repeated-measures ANOVA with time and treatment as variables. In experiment 2, differences between the five treatment groups at each time point were compared with one-way ANOVA, followed by post hoc analysis with Tukey's multiple comparison test. All the other measures were compared either by one-way ANOVA followed post hoc with Tukey's multiple comparison test or *t* test, as appropriate. Statistical analyses were performed using the Graph Pad Prism software version 3.00 for Windows (Graph Pad Software, San Diego, CA, U.S.A.). Significance level was set at $p < 0.05$.

RESULTS

Long-Term Effects of rAAV-lep Treatment in Prepubertal Rats

Leptin mRNA in the hypothalamus. Detectable leptin mRNA expression was observed in the hypothalami of untreated and rAAV-UF5-injected control female rats at 318 d of age (Fig. 1). However, leptin mRNA expression at this time was significantly higher than the control values in rats injected with rAAV-lep on postnatal d 24 (experiment 1).

BW and FI. In untreated, unoperated rats and in rats treated on d 24 with control virus encoding GFP, BW gain during the prepubertal and postpubertal interval was similar (Fig. 2A); each group displayed rapid weight gain until d 124, followed by a lower rate of gain until the end of the experiment at 318 d of age (294 d postinjection). Although a similar biphasic pattern of weight gain was also observed in rats receiving rAAV-lep on d 24, the rate of weight gain was attenuated starting at d 30, resulting in an overall BW that was 33–35%

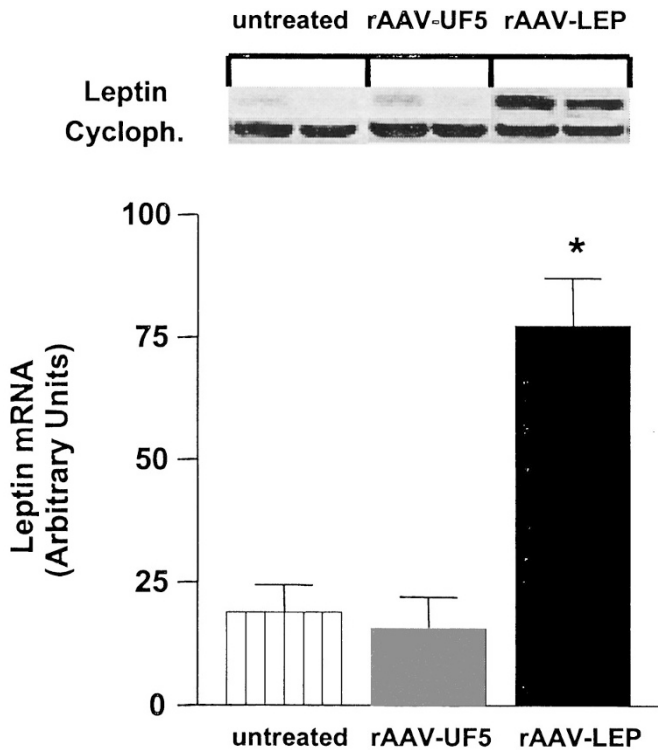


Figure 1. Analysis by reverse-transcriptase PCR analysis of hypothalamic leptin mRNA expression after 294 d of rAAV-lep treatment. * $p < 0.01$ vs rAAV-UF5 and untreated.

below that of the control groups through the course of experiment. Food consumption in the rAAV-UF5 control was also biphasic, initially a rapid exponential rise until d 54, followed by a near-plateau level of intake during the remainder of the

experiment (Fig. 2B). On the other hand, daily energy consumption in response to rAAV-lep injection was reduced by 23% from that seen in rAAV-UF5 controls during the entire course of the experiment. Inadvertently, FI was not monitored in the untreated group of rats in this experiment.

Serum hormones, metabolic variables, and BAT UCP-1 mRNA. In association with 33–35% reduction in BW (Fig. 2A) in rAAV-lep treated rats, serum leptin levels were reduced by 90% (Fig. 2C) and serum insulin by 72–76% from those found in the two control groups (Fig. 2D). Reduction in serum insulin in rAAV-lep-treated rats was accompanied by a small decrease (21–25%) in serum glucose levels ($p < 0.05$), but levels were still normoglycemic (85.2 mg/dL, Fig. 2E). rAAV-lep treatment significantly decreased serum FFA concentrations relative to those found in control rats ($p < 0.05$); serum ghrelin levels, however, were unaffected (Table 1). UCP-1 mRNA expression in BAT was similar in controls and rAAV-lep groups (Fig. 2F).

Short-Term Effects of rAAV-lep Treatment in Prepubertal Female and Male Rats

The aim of the next short-term experiment was to determine 1) whether the effects of short-term rAAV-lep treatment (89–111 d) were similar to those observed after 294 d postinjection; 2) whether icv rAAV-lep was similarly effective in prepubertal males; and 3) whether reduced food consumption alone was responsible for reduced serum hormonal and metabolic variables seen in rAAV-lep injected rats.

BW and FI. As shown in Figure 3, rAAV treatment in prepubertal females once again produced a significant reduction in BW (35–40%) and FI (18–19%) versus untreated and

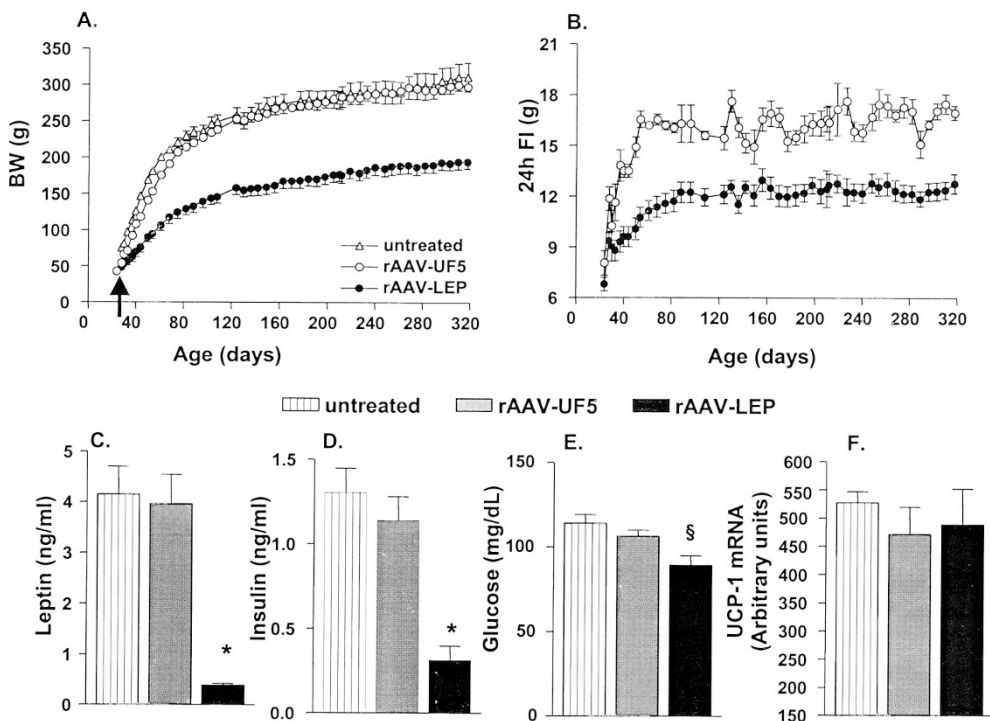


Figure 2. Effects of icv rAAV-lep injection on d 24 on BW (A) and FI (B) during 294 d postinjection (age 318 d). Serum leptin (C), insulin (D), glucose (E), and BAT UCP-1 mRNA expression (F) at 294 d postinjection. $\S p < 0.05$ and * $p < 0.001$ vs untreated and rAAV-UF5 controls.

Table 1. Effect of rAAV-lep injection on FFAs and ghrelin levels

Experiment 1		Untreated	rAAV-UF5	rAAV-lep		
Female	FFA (mEq/L)	0.86 ± 0.1	0.75 ± 0.1	0.43 ± 0.1*		
	Ghrelin (ng/mL)	0.47 ± 0.08	0.55 ± 0.03	0.42 ± 0.06		
Experiment 2		Untreated	Sham	rAAV-UF5	PF	rAAV-lep
Female	FFA (mEq/L)	0.46 ± 0.04	0.50 ± 0.06	0.42 ± 0.02	0.68 ± 0.08†	0.26 ± 0.03*
	Ghrelin (ng/mL)	0.33 ± 0.08	0.42 ± 0.04	0.48 ± 0.05	0.27 ± 0.06	1.46 ± 0.2*
Male	FFA (mEq/L)	0.64 ± 0.05	0.60 ± 0.03	0.63 ± 0.06	0.93 ± 0.05‡	0.46 ± 0.03*
	Ghrelin (ng/mL)	0.19 ± 0.01	0.19 ± 0.01	0.18 ± 0.03	0.33 ± 0.06§	0.35 ± 0.01**

FFA and ghrelin were analyzed in the serum obtained at the end of 294 d (Experiment 1), 89 d (Experiment 2, females), and 111 d (Experiment 2, males) of treatment. PF, pair-fed.

* $p < 0.05$ vs all the groups. ** $p < 0.01$ vs untreated, sham, rAAV-UF5, and PF groups. † $p < 0.05$ vs rAAV-UF5 and rAAV-LEP. ‡ $p < 0.01$ vs all the groups. § $p < 0.01$ vs untreated, sham, and rAAV-UF5-treated rats.

rAAV-UF5 ($p < 0.05$, Fig. 3, A and B), at the end of 89 d of observation. Likewise, rAAV-lep treatment in males (Fig. 4) decreased the weight gain and reduced daily food consumption (17–20% versus rAAV-UF5 and untreated controls, $p < 0.05$, Fig. 4, A and B) during the 111 d duration of the experiment.

Restriction of food consumption of prepubertal female and male PF rats to the amount consumed by their counterparts, rAAV-lep treated rats, resulted in a significant reduction in weight gain (Figs. 3A and 4A) but, in each instance, the weight reduction was significantly smaller than that displayed by the respective rAAV-lep-treated groups ($p < 0.05$).

Serum hormones, metabolic variables, and BAT UCP-1 mRNA. As in experiment 1 (Fig. 2), concomitant with the reduction in BW and FI, in female rats, serum leptin and insulin concentrations were drastically reduced in rAAV-lep group compared with controls (Fig. 3, C and D, $p < 0.001$). Interestingly, serum leptin and insulin concentrations were also

reduced in PF rats compared with control rats consuming *ad libitum* rat chow ($p < 0.001$). However, despite the equivalent energy consumption in rAAV-lep and PF groups, serum leptin and insulin concentrations were 2-fold lower in rAAV-lep group ($p < 0.05$), thereby implying that hypothalamic leptin overexpression was responsible for the exaggerated hormonal suppression. Serum glucose levels were slightly reduced in PF and in rAAV-lep-treated rats compared with those seen in control groups, but the levels were within the normal range (Fig. 3E). Interestingly, BAT UCP-1 mRNA was enhanced by rAAV-lep treatment compared with controls and PF groups, thereby indicating increased energy expenditure through thermogenesis.

In male PF rats, BW reduction was also significantly smaller than that in rAAV-lep-treated rats (Fig. 4A). Similarly, rAAV-lep treatment drastically reduced serum leptin and insulin concentrations compared with controls ($p < 0.001$). In con-

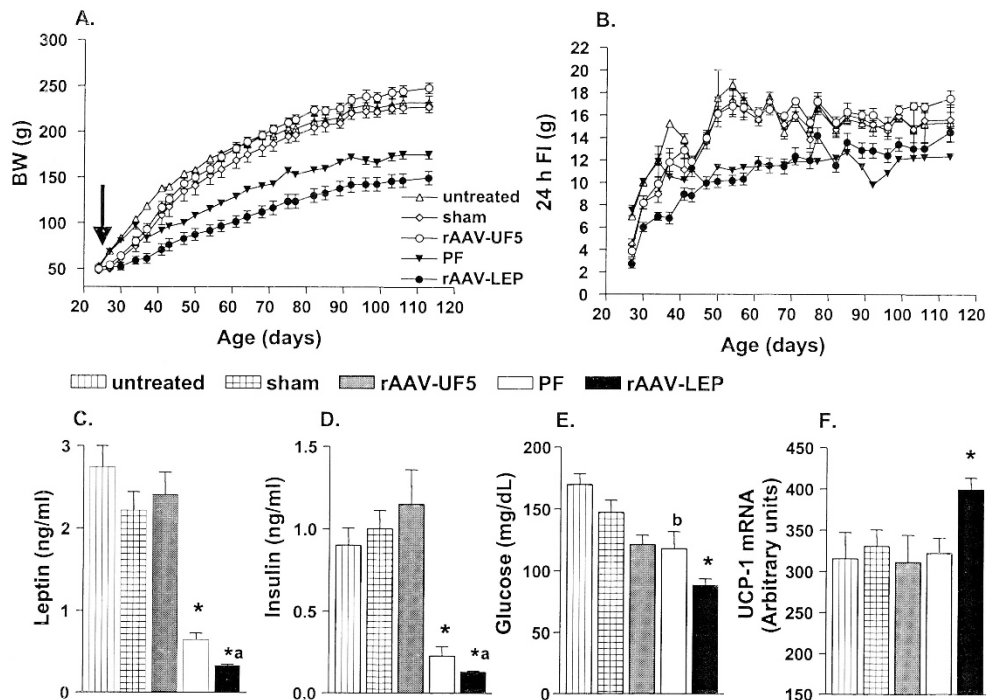


Figure 3. Effects of icv rAAV-lep injection on d 24 on BW (A) and FI (B) during 89 d postinjection in female rats (age 113 d). Serum leptin (C), insulin (D), glucose (E), and BAT UCP-1 mRNA expression (F) at 89 d postinjection. ^a $p < 0.05$ vs paired group; ^b $p < 0.05$ vs untreated group; * $p < 0.001$ vs untreated, sham, and rAAV-UF5-treated controls.

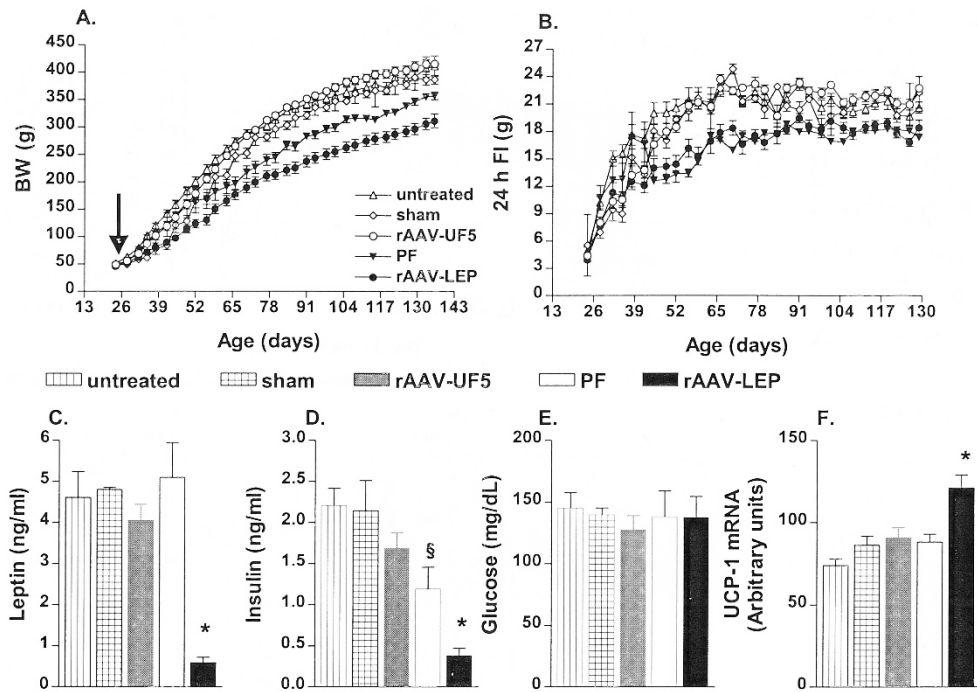


Figure 4. Effects of icv rAAV-lep injection on d 24 in male rats on BW (A) and FI (B) during 111 d postinjection (age 135 d). Serum leptin (C), insulin (D), glucose (E), and UCP-1 mRNA expression (F) in male rats at 111 d postinjection. * $p < 0.001$ vs untreated, sham, and rAAV-UF5 controls; § $p < 0.05$ vs untreated group.

trast, serum leptin was unchanged and insulin levels were only slightly altered in PF male rats. In rAAV-lep-treated male rats, serum glucose levels were in the range seen in control and PF groups, despite the drastic reduction in serum insulin. In male rats, rAAV-lep treatment also enhanced BAT UCP-1 mRNA expression over that observed in the control groups and the PF group.

Whereas serum FFA were reduced (Table 1, $p < 0.001$), serum ghrelin levels were elevated in both male and female rats after rAAV-lep injection on d 24 (Table 1). As reported previously (29), PF resulted in significantly higher FFA levels both in female and male rats, but serum ghrelin levels were elevated only in PF males and not in PF females.

Effect of rAAV-lep Injection on the Timing of Vaginal Opening and Reproductive Cycles

Between d 24 and 41 of age, the reduction in FI and BW failed to affect the timing of the onset of sexual maturation (Table 2). In the control as well as in rAAV-lep-treated rats, vaginal opening occurred between 35 and 41 d of age, as reported previously (39, 40). Although pair-feeding rats the amount equivalent to that consumed by rAAV-lep-treated rats decreased weight significantly less than that evoked by rAAV-lep treatment, it did not affect the timing of vaginal opening.

The impact on occurrence of regular estrous cycles subsequent to vaginal opening, as monitored at various intervals,

Table 2. Effect of rAAV-LEP injection on the time of the vaginal opening and the length of the estrous cycle

Day of vaginal opening					
	Untreated	Sham	rAAV-UF5	PF	rAAV-LEP
Experiment 1	38.6 ± 0.9	—	39.8 ± 0.9	—	39.9 ± 0.6
Experiment 2	34.8 ± 1.9	40 ± 0.9	39.6 ± 0.9	35.4 ± 2.4	38.6 ± 1.6
Number of estrous cycle (d)					
Age (d)	Untreated	Sham	rAAV-UF5	PF	rAAV-LEP
54–70*	3.2 ± 0.2	3.7 ± 0.3	3.3 ± 0.2	3.2 ± 0.2	3.3 ± 0.2
120–146	—	—	4.2 ± 0.3	—	3.9 ± 0.3
184–240	—	—	8.8 ± 1.0	—	8.4 ± 0.8
240–270	—	—	1.8 ± 0.6	—	2.3 ± 0.6
Length of estrous cycle (d)					
Age (d)	Untreated	Sham	rAAV-UF5	PF	rAAV-LEP
54–70*	5.3 ± 0.4	4.5 ± 0.4	5.1 ± 0.4	5.1 ± 0.3	5.3 ± 0.5
120–146	—	—	6.4 ± 0.5	—	6.9 ± 0.5
184–240	—	—	6.9 ± 1.1	—	6.4 ± 0.4
240–270	—	—	10.0 ± 3.5	—	15.8 ± 4.4

* Data on number of estrous cycles (d) and length of estrous cycles (d) collected during age 54–70 from experiments 1 and 2 were combined.

showed that the number and duration of estrous cycles were similar in rAAV-lep group and all control groups and PF rats. Also, as expected (46), estrous cycle length increased with age.

Effects of rAAV-lep Injection on Gene Expression of Hypothalamic Peptides Involved in Weight Homeostasis

The effects of prepubertal rAAV-lep injection on *NPY*, *AGRP*, and *POMC* mRNA expression in the hypothalamic ARC in adults are summarized in Figures 5 and 6. *NPY* but not *AGRP* mRNA expression was significantly decreased in the hypothalamic ARC of rAAV-injected compared with control groups at 294 d ($p < 0.05$, experiment 1, Figs. 5 and 6) and 89 d postinjection (experiment 2, Fig. 6). In contrast, *POMC* mRNA expression was enhanced in the hypothalamic ARC relative to controls in both experiments ($p < 0.05$, Figs. 5 and 6). Restriction of food intake by pair-feeding in short- and long-term experiments elicited opposite effects on neuropeptide expression. In PF rats, *NPY* mRNA expression increased and *POMC* mRNA decreased compared with that found in untreated and rAAV-UF5 rats fed chow *ad libitum* ($p < 0.05$). In addition, *AGRP* expression was augmented significantly in PF rats compared with controls and in rAAV-lep-treated rats ($p < 0.05$).

DISCUSSION

These results show that increased leptin transgene expression in the hypothalamus after administration of one icv injection of rAAV-lep to 24-d-old female and male rats evoked voluntary reduction in food intake and decreased the rate of weight gain during the entire pubertal and postpubertal periods of observation, lasting as long as 10 mo (experiment 1). To the

best of our knowledge, this is the first report of a long-lasting weight suppression by central leptin gene therapy initiated prepubertally. These findings complement the reported effectiveness of central rAAV-lep therapy in adult rats to produce sustained suppression of the age-dependent weight gain for 6 mo duration of experiment in rats consuming regular rat chow (11 Kcal %) (24, 25) and also the rapid weight gain and obesity in rats consuming a high-fat diet (45 Kcal %) (15).

Serum leptin concentrations were drastically reduced in rAAV-lep-treated rats in this study and in adult rats consuming regular rat chow or high-fat diet (15, 24, 25). Because shifts in leptin concentrations are reported to occur in direct proportion to fat depots in rodents and humans (1–3, 11–13), these low leptin levels, together with reduced adipose tissue-derived FFA, document a loss of fat. Leptin administration either systemically or centrally for short periods decreased adiposity without affecting lean body mass in leptin-deficient *ob/ob* mice and in normal outbred adult rodents (1–3). Central rAAV-lep therapy in adult rats also markedly decreased adiposity for extended periods without affecting lean tissue mass (25). Although not specifically analyzed in the current investigation, the possibility of prepubertal rAAV-lep treatment affecting lean tissue mass growth in freely feeding rats is quite remote.

Maintenance of reduced weight over extended periods most likely resulted from reduced energy intake along with increased thermogenic energy expenditure, possibly in conjunction with other sympathetic nervous system-driven energy disposition mechanisms (1, 2, 5), because an equivalent reduction in energy consumption alone in PF rats was far less effective in reducing weight. A significant increase in BAT UCP-1 mRNA in response to rAAV-leptin treatment was observed in both male and female rats in the short-term studies.

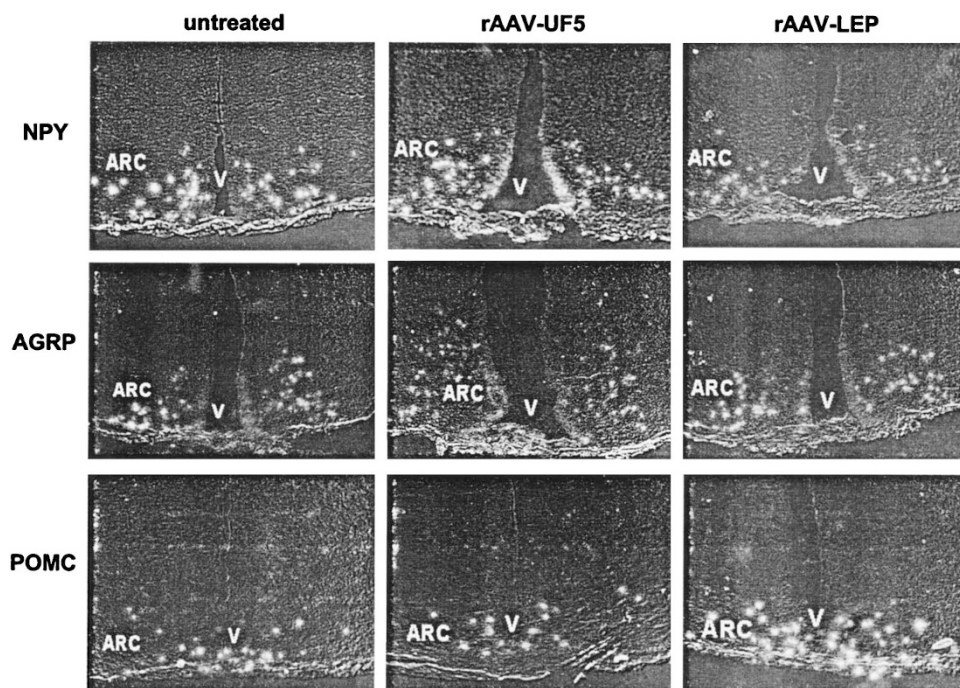


Figure 5. Representative microphotographs of NPY, AGRP and POMC mRNA expression in the ARC of untreated, rAAV-UF5, and rAAV-lep at 294 d postinjection (experiment 1). V, third ventricle.

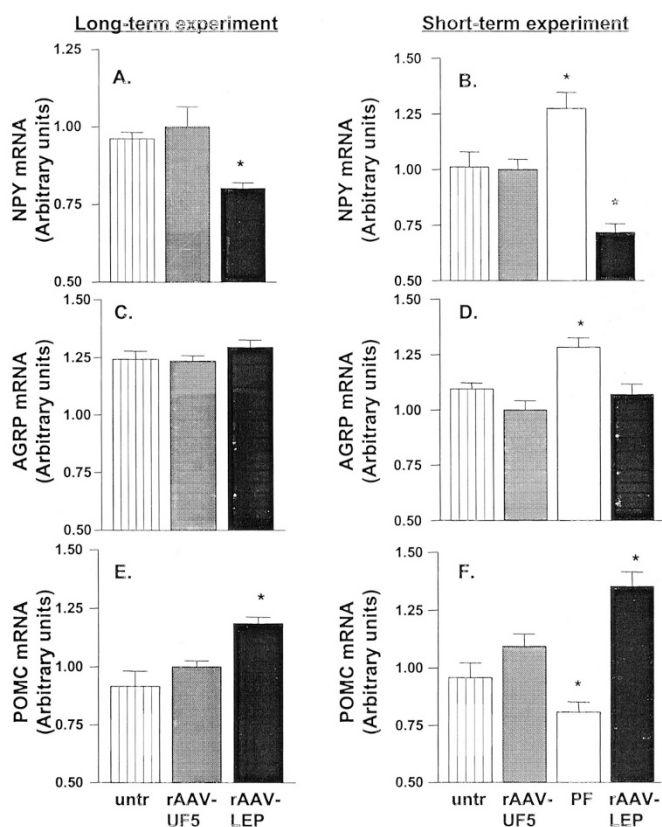


Figure 6. Effects of icv rAAV-lep treatment on NPY, AGRP, and POMC in female rats at 294 d (experiment 1, A, C, E) and 89 d post-treatment (experiment 2, B, D, F). * $p < 0.05$ vs all the groups.

This observation, along with analogous findings in adult rats (24, 25), is consistent with the inference that leptin overexpression in the hypothalamus suppresses weight gain in a manner quite similar to that reported after leptin injections for short periods in obese *ob/ob* mice and outbred normal rodents (1–3, 47). A new finding of the current study is that BAT UCP-1 mRNA was unchanged despite reduced food intake in older rats. This dichotomy, not apparent in adult rats observed for 6 mo (15, 24, 25), raises the intriguing possibility that the ability of hypothalamic leptin to suppress food intake is retained longer than its ability to augment energy expenditure, and that reduced energy consumption alone, at some time point in the postinjection period, can sustain low weight in older rats. The widely reported, age-related diminution in energy expenditure in rodents and humans (48) conforms to this possibility. Affirmation of our inference of increased energy expenditure, as reflected by BAT UCP1 mRNA, by direct calorimetry and characterization of the timing of onset as well as cellular and molecular basis of dichotomy in these two age-dependent central effects of leptin are underway.

That suppression of weight gain and appetite is a consequence of the relentless effects of sustained leptin overexpression in the hypothalamus is supported by the observation of increased leptin mRNA expression for 6 wk postinjection in adult rats (24) and for 10 mo postinjection in this study. Additionally, we have reported immunocytochemical localization of GFP, transduced from the control rAAV vector, in

several hypothalamic sites previously implicated in weight homeostasis (24, 25). Because peripheral leptin levels were drastically reduced in rAAV-lep-treated rats, suppression of food intake and augmented energy expenditure was due to the paracrine/autocrine action of locally produced leptin. Our findings clearly demonstrate a sustained weight-reducing effect of ectopic leptin into postpubertal period by a single central injection of rAAV-lep vector to prepubertal rats. This lack of insensitivity to centrally produced leptin for long periods contrasts with the well-known, rapid, age-related development of resistance to rising peripheral leptin, resulting in a loss of leptin's ability to inhibit weight gain and food intake (8–10). Seemingly, the age-related central leptin insufficiency results from defective transport of peripheral leptin to the hypothalamus and not the insensitivity of central targets to leptin itself, as advocated earlier (16–19, 24, 25).

In leptin-deficient obese *ob/ob* mice and humans, leptin replacement restored reproductive cyclicality (1–3, 49). Daily injections of leptin to normal outbred mice in the prepubertal period accelerated the onset of puberty (35, 36), however, subsequent studies failed to replicate these findings (37, 38). Leptin replacement prevented the delay in vaginal opening induced by severe caloric restriction either by fasting or an enforced severe reduction in caloric intake (>60%), paradigms that markedly decrease circulating leptin levels and weight (37, 39, 40). Thus, a permissive role for leptin in sexual maturation under normal conditions and a facilitatory role under severe caloric deficits is possible. In our experimental design, a reduction in caloric intake of about 22–31% during the prepubertal period and of about 19–25% postpubertally, either voluntarily, as in rAAV-lep-treated rats, or enforced, as in PF rats, affected neither the timing of vaginal opening nor the subsequent occurrence of regular estrous cycles. Also, the age-dependent reduction in number of estrous cycles and an increase in the duration of estrous cycles (46) was similar in control rats that displayed high circulating leptin and in rAAV-lep-injected rats with drastically diminished leptin levels. An important new outcome of our study is that the effects of voluntary or enforced reduced caloric intake on endocrine and neuroendocrine control of sexual maturation and reproductive cycles are different from those produced by fasting, a higher magnitude of caloric restriction (40, 41).

An analysis of both the short- and long-term impact of hypothalamic leptin overexpression on two functionally opposing, interconnected, appetite-regulating peptidergic neurons that express the biologically relevant long form of the leptin receptor (3–5) provides a neural basis for the sustained suppression of appetite, weight gain, and adiposity. The sustained decrease in *NPY* mRNA expression in the ARC implies an attenuated release of the appetite-stimulating neuropeptide in the PVN (5, 20). A concurrent increase in ARC *POMC* gene expression suggests enhanced release of the POMC-derived appetite inhibiting α -MSH in the PVN (1, 2, 5, 20). Consequently, this dual hypothalamic control on weight homeostasis, mediated by PVN targets, can account for the overall sustained reduction in motivational drive for food in rAAV-lep-injected rats. Similar effects on these two functionally opposing hypothalamic appetite-regulating signals were induced for 6 wk by

rAAV-lep treatment in adult rats with reduced energy consumption (24). The current study extends these findings to show that leptin overexpression locally in the hypothalamus continues to exert similar effects on NPY and POMC systems for as long as 10 mo duration of the experiment.

AGRP, an orexigenic peptidergic signal coexpressed with NPY in the ARC neurons, stimulates feeding by antagonizing the action of α -MSH at MC4 receptors in the PVN (5, 50). In this and our previous study (24), leptin transgene overexpression in the hypothalamus failed to affect ARC *AGRP* mRNA expression. In several other experimental paradigms, relatively negligible participation of AGRP in energy homeostasis has been reported (51). Our findings are in accord with the idea that AGRP signaling may not play a role in the current experimental design, and that locally produced leptin is capable of differentially modulating gene transcription in NPY and AGRP coexpressing neurons in the ARC. Inasmuch as several genes, in addition to *NPY* and *POMC*, play a role in the hypothalamic integration of energy homeostasis (5), it is likely that leptin overexpression in the hypothalamus may engage any one or more of these genes, in concert with NPYergic and melanocortin signalings, to produce a sustained appetite and body weight reduction.

There are three additional new outcomes of this study. First, leptin overexpression in the hypothalamus increased circulating ghrelin levels in younger female and male rats but not in older female rats. Second, this increase in serum ghrelin does not appear to be stimulated by reduced food intake because PF rats failed to display a similar consistent increase in this and the previous study (15). Although the factors responsible for increasing the release of ghrelin from the stomach (31, 32) are unknown at present, our results suggest that the stimulatory effects of ghrelin on appetite (32) were blocked by the robust anorectic effects of leptin overexpression in the hypothalamus. Furthermore, in older rAAV-lep-injected female rats, neither ghrelin levels nor BAT UCP1 mRNA expression were augmented. Whether impaired neural relay of information from the hypothalamus to peripheral targets alone or together with an age-dependent diminution in target responsiveness underlie these deficits remains to be ascertained.

Third, serum insulin levels were consistently and markedly reduced after rAAV-lep injection, a response accompanied by only a slight reduction in serum glucose in female rats in these and previous studies in female rats (15, 24, 25). In the current study, we find that serum glucose was unchanged in male rats. The decreases in serum insulin were consistently greater than those produced by restricted feeding in PF rats in these studies. Apparently, central leptin action effectively blocks the decrease in insulin sensitivity evident in control rats showing high serum insulin levels concomitant with normoglycemia. A similar blockade of the rapid development of insulin insensitivity was obtained in response to icv rAAV-lep in adult rats consuming either normal rat chow or high-fat diet (15, 24, 25). Low circulating insulin levels in association with diminished adiposity have been reported extensively (28, 29). It is plausible that an independent central action of leptin decreased pancreatic insulin release. Although leptin replacement in leptin-deficient obese *ob/ob* mice suppressed peripheral insulin levels

(52, 53), the role of peripheral leptin in regulating insulin release directly from pancreas in normal outbred rodents is controversial (28, 29, 54, 55). Furthermore, it was shown recently that peripheral hyperleptinemia was rendered ineffective in depleting fat and suppressing hyperinsulinemia in rats bearing lesions in the ventromedial hypothalamus that disrupted the outflow of information from the hypothalamus (56, 57). Seemingly in agreement with previous reports (15, 24, 23), the effects of peripheral hyperleptinemia to inhibit pancreatic insulin release and deplete fat are mediated centrally by mechanisms located in the hypothalamus.

In summary, these results show for the first time that one central injection of rAAV encoding leptin in prepubertal rats can transduce leptin transgene overexpression for 10 mo, the duration of the experiment. This persistent transgene overexpression decreases postpubertal weight gain accompanied by reduction in food intake, adiposity, and serum insulin and FFA. Suppression of weight gain and appetite apparently resulted from the relentless effects of leptin overexpression on hypothalamic genes regulating appetite. The increase in nonshivering thermogenic energy expenditure and plasma ghrelin levels, however, waned with age. Thus, central leptin gene therapy is a novel therapy to control postpubertal weight gain and adiposity for extended period in rodents and may be a therapeutic modality to ameliorate pediatric obesity.

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