Hypothalamic Leptin Gene Therapy Reduces Body Weight without Accelerating Age-Related Bone Loss

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Abstract

Excessive weight gain in adults is associated with a variety of negative health outcomes. Unfortunately, dieting, exercise, and pharmacological interventions have had limited long-term success in weight control and can result in detrimental side effects, including accelerating age-related cancellous bone loss. We investigated the efficacy of using hypothalamic leptin gene therapy as an alternative method for reducing weight in skeletally-mature (9-month-old) female rats and determined the impact of leptin-induced weight loss on bone mass, density, and microarchitecture, and serum biomarkers of bone turnover (CTx and osteocalcin). Rats were implanted with cannulae in the 3rd ventricle of the hypothalamus and injected with either recombinant adeno-associated virus encoding the gene for rat leptin (rAAV-Leptin, n=7) or a control vector encoding green fluorescent protein (rAAV-GFP, n=10) and sacrificed 18 weeks later. A baseline control group (n=7) was sacrificed at vector administration. rAAV-Leptin-treated rats lost weight (−4±2%) while rAAV-GFP-treated rats gained weight (14±2%) during the study. At study termination, rAAV-Leptin-treated rats weighed 17% less than rAAV-GFP-treated rats and had lower abdominal white adipose tissue weight (−80%), serum leptin (−77%), and serum IGF1 (−34%). Cancellous bone volume fraction in distal femur metaphysis and epiphysis, and in lumbar vertebra tended to be lower (p<0.1) in rAAV-GFP-treated rats (13.5-months-old) compared to baseline control rats (9-months-old). Significant differences in cancellous bone or biomarkers of

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bone turnover were not detected between rAAV-Leptin and rAAV-GFP rats. In summary, rAAV-Leptin-treated rats maintained a lower body weight compared to baseline and rAAV-GFP-treated rats with minimal effects on bone mass, density, microarchitecture, or biochemical markers of bone turnover.

Keywords

rAAV-Leptin; microcomputed tomography; dual energy absorptiometry; white adipose tissue

Introduction

Insidious excessive weight gain is common in adults and is associated with increased risk for various chronic diseases, including heart disease, chronic obstructive pulmonary disease, type 2 diabetes, osteoarthritis, and certain cancers (Franssen, et al. 2008; Low, et al. 2009; Magliano 2008). Weight loss, whether induced by caloric restriction alone or in combination with exercise and/or pharmaceutical intervention, may attenuate or reverse the health risks associated with excessive weight gain. Unfortunately, the long-term efficacy of conventional weight loss interventions is generally poor and many individuals weight-cycle through repetitive bouts of weight loss followed by rapid weight regain (Elfhag and Rossner 2005; Wu, et al. 2009; Yaskin, et al. 2009).

The adipokine leptin plays an essential role in energy homeostasis (Rosenbaum and Leibel 2014) and adult-onset weight gain is closely associated with an increase in circulating leptin and development of leptin resistance (Carter, et al. 2013; Knobelspies, et al. 2010; Morris and Rui 2009; Scarpace and Tumer 2001). As a consequence, avoiding leptin resistance may be essential to achieving life-long weight control. However, the mechanisms mediating leptin resistance are incompletely understood. To date, studies suggest that high blood leptin concentrations result in saturation of leptin transport across the blood brain barrier (blood brain barrier resistance) (Banks and Lebel 2002) and/or down regulation of leptin signaling in the hypothalamus due to constitutive expression of suppressor of cytokine signaling (hypothalamic resistance) (Bjorbaek, et al. 1998).

Delivery of leptin directly into the hypothalamus by gene therapy normalizes body weight and extends lifespan in morbidly obese leptin-deficient *ob/ob* mice in the absence of detectable circulating leptin (Boghossian, et al. 2007; Dhillon, et al. 2000). Additionally, hypothalamic leptin gene therapy slows weight gain in rodents capable of producing leptin (Boghossian, et al. 2005). These findings suggest that increasing leptin levels directly in the hypothalamus, in addition to bypassing blood brain barrier-mediated leptin resistance, overcomes hypothalamic leptin resistance. However, it is less clear whether this approach can induce weight loss and/or maintain lower body weight in aging rodents with normal circulating levels of the hormone (Shapiro, et al. 2008).

In humans, calorie restriction-induced weight loss is often associated with bone loss, leading to increased risk for osteoporosis (Lee, et al. 2010b; Shapses and Riedt 2006). Importantly, weight regain does not restore bone and, as a consequence, weight cycling is especially deleterious to the skeletal system (Lee et al. 2010b; Villalon, et al. 2011). Because

osteoporotic fractures are associated with decreased quality of life and increased mortality (Cauley 2013), there is strong incentive to develop weight loss strategies that preserve bone mass.

Leptin is a candidate factor for coupling bone metabolism to energy availability. Leptin is required for normal skeletal growth, maturation and turnover (Arounleut, et al. 2013; Gat-Yablonski and Phillip 2008; Turner, et al. 2013b; Turner, et al. 2014). Weight loss results in decreased leptin levels (Hamann and Matthaei 1996), reduced bone accrual during growth (Devlin, et al. 2010), and accelerated age-related bone loss (Talbott, et al. 2001; Turner and Iwaniec 2011). Importantly, leptin treatment attenuates the inhibitory effects of caloric restriction on bone growth (Gat-Yablonski, et al. 2004; Goldstone, et al. 2002). Furthermore, leptin administered at supraphysiological levels has been reported to maintain bone mineral density despite inducing weight loss (Stunes, et al. 2012).

Taken together, the above findings suggest the respective skeletal changes accompanying caloric restriction-induced weight loss and leptin-induced weight loss differ. Specifically, low hypothalamic leptin levels induced by caloric restriction lead to an adaptive response similar to starvation and result in rapid bone loss. In contrast, elevating hypothalamic leptin levels decreases appetite and increases energy expenditure (Friedman 2010) leading to weight loss with minimal impact on the skeleton. Although suggestive, the aforementioned conclusions are based on short-term studies where leptin was administered subcutaneously to growing rats with relatively low levels of circulating leptin. It is less clear whether similar benefits can be achieved in skeletally mature animals exhibiting weight gain as well as age-related bone loss. As such, the present study was designed to determine the long-term efficacy of increased hypothalamic leptin in reducing adult onset-associated weight gain and the impact this intervention has on bone mass, density and architecture.

Material and Methods

Animals

Nine-month-old female Sprague Dawley rats (Harlan; Indianapolis, IN) were used in the experiment. The rats were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals and the experimental protocol was approved (IACUC #D642) by the Institutional Animal Care and Use Committee at the University of Florida (Gainesville, FL).

Experimental Design

The rats were randomized by weight into 3 groups: baseline control (n = 7), rAAV-GFP control (n = 10), and rAAV-Leptin (n = 7). The baseline group (body weight; 289 ± 7g, mean ± SE) was sacrificed at vector administration. The rAAV-GFP and rAAV-Leptin groups were sacrificed at 18 weeks post-vector administration. All rats were housed individually in a temperature- (21–23°C) and light-controlled (lights on 8:00 – 18:00 hours) room under specific pathogen-free conditions. Food and water were available *ad libitum* to all animals. The rats were weighed and food consumption determined weekly.

Construction and Packaging of rAAV Vectors

The rAAV-Leptin and rAAV-GFP vectors were constructed and packaged as described previously (Beretta, et al. 2002). In brief, the vector pTR-CBA-Ob EcoRI fragment of pCR-rOb (a gift from Dr. Roger H. Unger, University of Texas Southwestern Medical Center, Dallas, TX) containing rat leptin cDNA was subcloned into rAAV vector plasmid pAAVβGEnh after deleting the EcoRI fragment carrying the β-glucoronidase cDNA sequence (Dhillon 2000; Dhillon et al. 2000; Dhillon, et al. 2001a; Zolotukhin, et al. 1999). The control vector, rAAV-GFP, was similarly constructed to encode the GFP gene (Dhillon 2000; Dhillon et al. 2001a; Dube, et al. 2002; Zolotukhin et al. 1999).

Vector Administration

For vector administration, the rats were anesthetized with 2–3% isoflurane delivered in oxygen and stereotaxically implanted with a permanent cannula in the 3rd cerebroventricle. The coordinates employed for cannula placement were based on the rat brain atlas. After 1 week of recovery, rats were injected once intracerebroventricularly with either rAAV-GFP (5 μL, 8.25×10^{11} virus particles) or rAAV-Leptin (5 μL, 7.7×10^{11} virus particles).

Tissue Collection and Analyses

Rats were fasted overnight prior to tissue collection. For tissue collection, the rats were anesthetized with 2–3% isoflurane delivered in oxygen. Blood was collected from abdominal aorta and serum stored at −20°C for analysis of leptin, insulin-like growth factor 1 (IGF1), growth hormone, glucose, adiponectin, collagen type 1 cross-linked C-telopeptide (CTx), and osteocalcin. Death was induced by decapitation. Hypothalami were excised and stored in RNAlater (Ambion, Austin, TX) for analysis of leptin and NPY mRNA levels. Abdominal white adipose tissue (WAT) was excised and weighed and samples of WAT were stored in RNAlater for analysis of leptin and IGF1 mRNA levels. Femora and 2nd lumbar vertebrae were collected and stored in 70% ethanol for analysis of bone mass, density, and architecture.

Serum Chemistry

Serum glucose was measured using Autokit Glucose (Wako, Richmond, VA). Serum leptin was assayed using the rat leptin radioimmunoassay kit from Linco Research, Inc. (St Louis, MO) according to the manufacturer's instructions. Serum IGF1 was measured with a radioimmunoassay using a polyclonal antibody to IGF1 after separation of IGF-binding proteins by acid ethanol extraction. Serum osteocalcin was measured using rat Gla-osteocalcin high sensitive EIA kit (Clontech, Mountain View, CA). Serum CTx was measured using rat CTx-I ELISA kit (Novateinbio, Cambridge, MA). Serum adiponectin was measured using rat total adiponectin Quantikine ELISA kit (R&D Systems, Minneapolis, MN). Serum growth hormone was measured using a rat/mouse growth hormone ELISA kit (EMB Millipore, Billerica, MA).

RNA analysis

Tissue samples were homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA). Total cellular RNA was isolated according to manufacturer's protocol. RNA quantity was

determined spectrophotometrically, and RNA quality was evaluated via formaldehyde agarose gel electrophoresis.

Real-Time PCR

cDNA for RT-PCR was synthesized using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). 1 μg of total RNA was reverse transcribed using random hexamer primers and SuperScript II reverse transcriptase according to manufacturer's protocol. Real-time PCR primers that are specific for rat leptin (forward: 5′-CCTGTGGCTTTGGTCCTATCTG-3′, reverse: 5′-AGGCAAGCTGGTGAGGATCTG-3′), NPY (forward′ 5′-GCCATGATGCTAGGTAACAAACG-3′, reverse: 5′-GTTTCATTTCCCATCACCACATG-3′), IGF1 (forward: 5′-CCGGACCAGAGACCCTTTG-3′, reverse: 5′-CCTGTGGGCTTGTTGAAGTAAAA-3′), and 18S ribosomal RNA (18S) (forward: 5′-GGACCAGAGCGAAAGCATTTGC-3′, reverse: 5′-CGCCAGTCGGCATCGTTTATG-3′) were synthesized by Operon Biotechnologies (Huntsville, AL). Real-time PCR reactions were performed using DyNAmo HS SYBR Green qPCR kit (New England Biolabs, Ipswich, MA). A standard curve that was generated from serial dilutions of purified plasmid DNA that encoded the respective genes was used to measure mRNA transcript copy number. mRNA data represent normalized copy number using 18S ribosomal RNA gene.

Dual Energy X-ray Absorptiometry

Total femur bone mineral content (BMC, g), area (cm^2), and bone mineral density (BMD, g/cm^2) were measured *ex vivo* using dual energy absorptiometry (DXA; Piximus 2, Lunar Corp., Madison, WI).

Microcomputed Tomography

Microcomputed tomography (μCT) was used for nondestructive high resolution 3-dimensional evaluation of cortical and cancellous bone volume and architecture. Midshaft and distal femora and 2nd lumbar vertebrae were scanned in 70% ethanol using a Scanco μCT40 scanner (Scanco Medical AG, Basserdorf, Switzerland) at a voxel size of $16 \times 16 \times 16$ μm (55 kV_p x-ray voltage, 145 μA intensity, and 200 ms integration time). Filtering parameters sigma and support were set to 0.8 and 1, respectively. Bone segmentation was conducted at a threshold of 245 (scale, 0–1000) determined empirically. Cortical bone in the mid femur diaphysis and cancellous bone in the distal femur metaphysis and epiphysis were evaluated. Automated contouring was used to delineate cortical bone from non-bone. Following, all cortical slices were examined visually for inclusion of cancellous struts originating from the endocortex (extremely rare at this site) and manually removed when present. Twenty consecutive slices (320 μm) of bone were evaluated and cross-sectional volume (cortical and marrow volume, mm^3), cortical volume (mm^3), marrow volume (mm^3), and cortical thickness (μm) were measured. Polar moment of inertia (I_{Polar}) was determined as a surrogate measure of bone strength in torsion. For the femoral metaphysis, 75 consecutive slices (1,200 μm) of cancellous bone, 150 slices (2400 μm) proximal to the growth plate, were evaluated. The entire cancellous compartment (60 ± 1 slices, 960 ± 16 μm, mean ± SE) was assessed in the femoral epiphysis. Analysis of lumbar vertebra included the entire region of cancellous bone between the cranial and caudal growth plates (282 ± 2

slices, 4,512 ± 36 μm). Manual contouring was used to delineate cancellous from cortical bone in the femur metaphysis, femur epiphysis, and vertebral body. Direct cancellous bone measurements included cancellous bone volume fraction (bone volume/tissue volume, %), connectivity density (mm^{-3}), trabecular thickness (μm), trabecular number (mm^{-1}), and trabecular spacing (μm) (Thomsen, et al. 2005).

Statistical Analysis

Mean responses were compared between three groups (baseline, rAAV-GFP, and rAAV-Leptin) using analysis of variance (ANOVA), while two-group comparisons were made using t-tests. A modified F test was used when the assumption of equal variance was violated, with Welch's two-sample t-test used for two-group comparisons (Welch 1951). The Kruskal-Wallis nonparametric test was used when only the normality ANOVA assumption was violated, in which case the Wilcoxon-Mann-Whitney test was used for two-group comparisons. The required conditions for valid use of t-tests and ANOVA were assessed using Levene's test for homogeneity of variance, plots of residuals versus fitted values, normal quantile plots, and the Anderson-Darling test of normality. Longitudinal data on weekly measurements of body weight (weeks 0 to 18) and food intake (weeks 1 to 18) were analyzed using linear mixed models to account for correlated data. To accommodate different slopes for the rAAV-GFP and rAAV-Leptin groups and varying slope coefficients across time for these groups, the data on body weight were modeled using a random intercept linear spline model with a single knot at week 4. The random intercept, random slope mixed model for food intake allowed for different marginal intercepts and slopes between the two groups. The Benjamini and Hochberg method for maintaining the false discovery rate at 5% was used to adjust for multiple comparisons (Benjamini and Hochberg 1995). Differences were considered significant at p   0.05. Data are presented as mean ± SE. Data analysis was performed using R version 2.12 (Team 2010).

Results

The effects of hypothalamic rAAV-Leptin gene therapy on body weight, abdominal WAT weight, and food intake are shown in Figure 1. rAAV-Leptin-treated rats lost weight (-4 ± 2%) during the 18 week duration of study; the estimated decrease in mean weight from weeks 0 to 18 was 8 ± 3 grams (p = 0.006; 95% CI: 2 grams, 13 grams). The weight loss in the rAAV-Leptin group occurred during the initial 4 weeks post-vector administration (estimated slope: −3.6, p < 0.0001; 95% CI: −5.2, −2.1) with weight increasing thereafter (estimated slope: 0.5, p = 0.01; 95% CI: 0.12, 0.85) (Figure 1A and B). In contrast, control rAAV-GFP-treated rats gained weight (14 ± 2%) during the study; the estimated increase in mean weight from weeks 0 to 18 was 40 ± 2 grams (p = 0.006; 95% CI: 36, 45). The increase in weight was greater during the first 4 weeks following vector administration (estimated slope: 7.7, p < 0.0001; 95% CI: 6.4, 9.0) than during the subsequent 13 weeks (estimated slope: 0.7, p < 0.0001; 95% CI: 0.39, 1.01). The rate of change in body weight (i.e., slope) differed between rAAV-GFP-treated and rAAV-Lep-treated rats during the first 4 weeks of treatment (p < 0.0001) but not during the subsequent 13 weeks. At study termination, rAAV-Leptin-treated rats weighed 17% less (53 ± 10 grams; p < 0.0001; 95% CI: 32 grams, 74 grams) than the rAAV-GFP-treated rats. This difference in body weight

was associated with a pronounced reduction in abdominal WAT; WAT weight was 80% lower in rAAV-Leptin-treated compared to rAAV-GFP-treated rats (Figure 1C).

Food consumption was lower in rAAV-Leptin-treated rats compared to rAAV-GFP-treated rats (Figure 1D). Although significant differences in food intake were not detected between the two groups at 1 week post-vector administration, the estimated difference in mean food intake between the two groups during weeks 2 to 4 was 2.4 g/d (95% CI: 1.6, 3.3; p<0.0001); food intake averaged 17.1 g/d (95% CI: 16.2, 17.9) in the leptin group and 19.5 g/d (95% CI: 18.8, 20.2) in the GFP group (p<0.0001). For weeks 5 to 18, the difference in food intake was still significant (p=0.002), but the estimated difference dropped to 1.3 g/d (95% CI: 0.5, 2.1); food intake averaged 17.2 g/d (95% CI: 16.4, 17.9) in the leptin group and 18.5 g/d (95% CI: 17.86, 17.90) in the GFP group. Total food intake over the 18 week duration was 9% lower (p=0.071) in the rAAV-Leptin group compared to the rAAV-GFP group (Figure 1E).

The effects of rAAV-Leptin gene therapy on leptin and NPY mRNA expression in hypothalamus and leptin and IGF1 mRNA expression in abdominal WAT are shown in Figure 2. As expected, rAAV-Leptin-treated rats had higher hypothalamic leptin mRNA levels than rAAV-GFP-treated rats (Figure 2A). Significant differences in NPY mRNA expression in hypothalamus were not detected with treatment (Figure 2B). Significant differences were also not detected with treatment for leptin and IGF1 mRNA expression in abdominal WAT (Figure 2C and D).

The effects of rAAV-Leptin gene therapy on serum leptin, IGF1, growth hormone, glucose, adiponectin, CTx, and osteocalcin are shown in Figure 3. Serum leptin and IGF1 concentrations were lower in rAAV-Leptin-treated rats compared to rAAV-GFP-treated rats (Figure 3A and B). Significant differences between rAAV-Leptin-treated and rAAV-GFP-treated rats were not detected for growth hormone (Figure 3C), glucose (Figure 3D), or adiponectin (Figure 3E). Furthermore, significant differences between the 2 treatment groups were not detected for CTx, a marker of bone resorption (Figure 3F) or osteocalcin, a marker of bone turnover (Figure 3G).

The effects of rAAV-Leptin gene therapy on femur area, BMC, and BMD are shown in Figure 4. Bone area tended to be higher in rAAV-GFP-treated rats (13.5 months old) compared to baseline control rats (9 months old; p = 0.072) and rAAV-Leptin-treated rats (13.5 months old; p = 0.056). Significant differences in bone area were not detected between rAAV-Leptin-treated rats and baseline control rats. Significant differences in either BMC or BMD were not detected among baseline, rAAV-GFP, and rAAV-Leptin rats.

The effects of rAAV-Leptin gene therapy on cortical bone in the femoral diaphysis and cancellous bone in the femoral metaphysis and epiphysis are shown in Table 1.

Femur diaphysis

13.5-month-old rAAV-GFP-treated rats had greater cortical volume and tended to have greater polar moment of inertia (p = 0.072) than 9-month-old baseline control rats. Cross-sectional volume, cortical volume, and polar moment of inertia also tended to be greater (p =

0.058, p = 0.071, and p = 0.056, respectively) in rAAV-GFP-treated rats compared to rAAV-Leptin-treated rats. Significant differences between 13.5-month-old rAAV-Leptin-treated rats and 9-month-old baseline control rats were not detected for any of the cortical endpoints evaluated. Significant differences in marrow volume and cortical thickness were not detected among the 3 groups.

Distal femur metaphysis

13.5-month-old rAAV-GFP-treated rats had lower connectivity density and tended to have lower cancellous bone volume fraction (p = 0.071) and trabecular number (p = 0.056) and greater trabecular spacing (p = 0.072) than 9-month-old baseline control rats. 13.5-month-old rAAV-Leptin-treated rats tended to have lower connectivity density (p = 0.060) and trabecular number (p = 0.072) and greater trabecular spacing (p = 0.084) than 9-month-old baseline control rats. Significant differences between rAAV-GFP and rAAV-Leptin rats were not detected for any of the cancellous endpoints evaluated. Significant differences in trabecular thickness were not detected among the 3 groups.

Distal femur epiphysis

Cancellous bone volume fraction tended to be lower (p = 0.056) in 13.5-month-old rAAV-GFP-treated rats and was lower in 13.5-month-old rAAV-Leptin-treated rats than in 9-month-old baseline control rats. Significant differences in cancellous bone volume fraction were not detected between rAAV-GFP-treated and rAAV-Leptin-treated rats. Significant differences in connectivity density, trabecular number, trabecular thickness, and trabecular spacing were not detected among the 3 groups.

Lumbar vertebra

The effects of rAAV-Leptin gene therapy on cancellous bone in the lumbar vertebra are shown in Table 2. 13.5-month-old rAAV-GFP-treated rats tended to have lower cancellous bone volume fraction (p = 0.071) and trabecular number (p = 0.072) and greater trabecular spacing (p = 0.072) than 9-month-old baseline control rats. Significant differences in bone volume fraction, trabecular number, or trabecular spacing were not detected between rAAV-GFP-treated and rAAV-Leptin-treated rats. Significant differences among the 3 groups were not detected for connectivity density or trabecular thickness.

Discussion

The long-term effects of hypothalamic rAAV-Leptin gene therapy on energy balance and bone metabolism were evaluated in skeletally mature (9-month-old) female rats. Consistent with previous studies (Boghossian et al. 2005), rAAV-Leptin-treated rats lost weight while rAAV-GFP-treated rats gained weight during the 18 week duration of study. The respective changes in weight occurred during the first few weeks following vector administration, stabilizing thereafter. At study termination, rAAV-Leptin-treated rats weighed less than rAAV-GFP-treated rats and had lower abdominal WAT weight, serum leptin, and serum IGF1. In contrast, rAAV-Leptin treatment had minimal long-term effects on bone mass, density, and architecture or serum markers of bone turnover.

In the present study, the initial reduction in body weight following leptin gene therapy was associated with lower (−12.3%) energy intake compared to rAAV-GFP treatment. The initial increase in energy intake in rAAV-GFP-treated rats following vector administration likely represents a rebound in food consumption following a surgery-induced reduction in food intake. In support, historical data indicate that age-matched *ad lib*-fed rats consume ~18 g/d of diet (Iwaniec, et al. 2011). In contrast, an attenuated rebound in food intake was apparent in rAAV-Leptin treated rats. Once weight stabilized at a lower level, energy intake continued to be lower (−7%) in the rAAV-Leptin-treated rats than in rAAV-GFP-treated rats. These findings are consistent with previous long-duration gene therapy studies (Dhillon et al. 2001a; Torto, et al. 2006). The regulatory actions of leptin to reduce energy intake are mediated through a hypothalamic relay involving activation of leptin receptors on orexigenic NPY and anorectic proopiomelanocortin-expressing neurons (Forbes, et al. 2001; Schwartz, et al. 1996b). Morbidly obese leptin-deficient *ob/ob* mice are hyperphagic and express increased hypothalamic mRNA levels for NPY (Schwartz, et al. 1996a) and these abnormalities are reversed following leptin treatment (Duan, et al. 2007). At study termination, hypothalamic leptin gene expression was elevated in the rAAV-Leptin gene therapy group but significant differences in hypothalamic NPY gene expression were not observed between treatment groups. It is possible, however, that reduced NPY expression was associated with initial weight loss following vector administration (Bagnasco, et al. 2002; Beretta et al. 2002).

Genetic obesity in leptin-deficient *ob/ob* mice is reversed following administration of leptin (Levin, et al. 1996). In contrast, normal rats and mice exhibit age-related weight gain in spite of increased leptin levels. The failure of increased serum leptin to prevent further weight gain is generally attributed to blood brain barrier leptin resistance and/or hypothalamic leptin resistance (Banks, et al. 1999; Banks, et al. 1996; Bluher and Mantzoros 2004; Bray 2004; Burguera, et al. 2000; Frederich, et al. 1995; Sahu 2002; Van Heek, et al. 1997). Gene therapy bypasses blood brain barrier leptin resistance by direct introduction of leptin into the hypothalamus. The present study demonstrates the long-term efficacy of this approach in inducing weight loss and maintaining lower body weight in skeletally mature female rats. Factors such as diet composition and physical activity may impact the efficacy of hypothalamic leptin gene therapy in preventing excessive weight gain (Dube et al. 2002; Dube, et al. 2008; Shapiro et al. 2008). Nevertheless, our findings in rats consuming normal diet suggest that increasing hypothalamic leptin levels overcomes leptin resistance to prevent adult-onset weight gain.

The elevated leptin mRNA levels that we detected in the hypothalamus 18 weeks following administration of rAAV-Leptin contrast with the notable reduction in serum leptin. The 77% reduction in serum leptin observed in the present study was associated with a similar decrease (−80%) in abdominal WAT weight. This finding is consistent with the concept that leptin levels reflect fat stores (Hickey, et al. 1996). It is notable that the magnitude of lowering WAT weight (−80%) following rAAV-Leptin treatment greatly exceeded lowering body weight (−17%). This is consistent with previous reports indicating that rAAV-Leptin treatment preferentially reduces adipose tissue while preserving lean tissue (Dhillon, et al. 2001b). Furthermore, leptin administration has been shown to increase muscle mass in

leptin–deficient *ob/ob* mice (Bartell, et al. 2011) and aged C57BL6 mice (Hamrick, et al. 2010).

rAAV-Leptin treatment lowered serum IGF1 levels. IGF1 is an important mediator of the anabolic actions of growth hormone. A primary target of growth hormone is the liver, where the hormone stimulates IGF1 secretion through the activation of the hepatic growth hormone receptor. In turn, IGF1 modifies growth hormone secretion through a negative-feedback loop (Clemmons 2007). Increased IGF1 levels and decreased growth hormone secretion are associated with obesity (Garten, et al. 2012), whereas decreased IGF1 levels are associated with caloric restriction (Mitterberger, et al. 2011; Rasmussen, et al. 1995). Thus, the observed decrease in serum IGF1 could reflect an increase in growth hormone secretion. However, we did not detect a change in growth hormone levels with treatment, a finding consistent with the lack of an effect of caloric restriction on growth hormone levels reported in obese subjects in some human studies (Mitterberger et al. 2011; Rasmussen et al. 1995).

A substantial proportion of serum IGF1 is nonhepatic in origin (Liu, et al. 2000). Adipocytes produce IGF1 (D'Esposito, et al. 2012), potentially contributing to reduced growth hormone secretion associated with obesity. Leptin gene therapy did not impact IGF1 mRNA levels in WAT but the dramatic decrease in WAT following gene therapy may have contributed to the reduction in serum levels of this growth factor.

The decrease in serum IGF1 following rAAV-Leptin administration in normal rats contrasts with the response of *ob/ob* mice to leptin. Whereas, acute intravenously administered leptin had no effect on serum IGF1 (Burcelin, et al. 1999), chronic intracerebroventricular leptin administration to *ob/ob* mice was reported to increase serum IGF1 levels (Bartell et al. 2011). The latter long-term response may be related to normalization of growth hormone secretion in *ob/ob* mice (Luque, et al. 2007; Sinha, et al. 1975).

Elevated blood leptin is a risk factor for cardiovascular disease, diabetic kidney disease and several cancers (Cheung, et al. 2010; Cleary, et al. 2010; Hoda, et al. 2012; Hou and Luo 2011; Nishimura, et al. 2012) and high serum IGF1 levels are associated with increased cancer risk (Chaves and Saif 2011; Cleary, et al. 2009; Ribeiro, et al. 2004). Thus, reduction in serum leptin and IGF1, in addition to weight loss, may be a positive side effect of increasing hypothalamic leptin (Lee, et al. 2010a; Moore, et al. 2008; Olivo-Marston, et al. 2009; Wu, et al. 2010).

Leptin increases bone nodule formation *in vitro* (Gordeladze, et al. 2002) and increases bone formation when delivered to *ob/ob* mice by either intermittent subcutaneous administration (Turner et al. 2013b) or intracerebroventricular infusion (Bartell et al. 2011). Furthermore, hypothalamic leptin gene therapy increases osteoblast-lined bone perimeter and serum osteocalcin, and normalizes bone architecture in *ob/ob* mice without increasing serum leptin (Turner et al. 2013b). Although *ob/ob* mice have reduced osteoblast number and activity, the effects of leptin deficiency on bone metabolism appear to be partially compensated for by factors related to development of morbid obesity. Specifically, normalizing weight gain in *ob/ob* mice by dietary restriction and thermoneutral housing accentuated their skeletal

phenotype (Turner et al. 2014), characterized by reduced bone growth and turnover, low total bone mass and mild osteopetrosis.

In contrast to the notable effects of leptin to increase bone formation in leptin-deficient mice, increased leptin levels associated with moderate weight gain had no effect on bone metabolism in skeletally mature rats (Turner and Iwaniec 2010). Similarly, prevention of weight gain in skeletally mature rats by mild caloric restriction had no negative impact on bone formation (Turner and Iwaniec 2010). In the present long-term study, reduced serum leptin induced by hypothalamic leptin gene therapy was not associated with altered serum markers of bone turnover or accelerated age-related bone loss. Taken together, these findings suggest that the bone anabolic effects of leptin are most pronounced at low serum concentrations of the hormone and as a consequence hyperleptinemia does not confer additional positive skeletal benefits.

Bone mass in growing rodents is tightly coupled to body size (Iwaniec, et al. 2009). Cessation of linear bone growth occurs in rats as a consequence of formation of bone bridges that penetrate the growth plate, rendering further growth unfeasible (Martin, et al. 2003). The time table for linear growth cessation varies among bones but growth generally ceases in female Sprague Dawley rats by 8 months of age. As in adult humans, periosteal bone formation continues in long bones of skeletally mature rats at a very slow rate throughout life (Briot, et al. 2010; Stathopoulos, et al. 2011). Weight typically increases in rodents following skeletal maturity but the impact of weight changes on the skeleton has not been thoroughly explored (Iwaniec and Turner 2013). In the present study, bone area measured by DXA and cortical bone volume measured by µCT exhibited an upward trend in rAAV-GFP rats whereas these parameters remained at baseline control levels in rAAV-Leptin rats. The strong association between bone size and body weight typically observed in WT mice are preserved in leptin-deficient *ob/ob* mice (Iwaniec et al. 2009), a finding suggesting that increased bone size in aging rAAV-GFP rats is due to their increased body weight.

As expected, age-related cortical bone loss was not observed in the present study. In humans, a negative intracortical bone remodeling balance contributes to age-related bone loss (Seeman 2013). A limitation of rodents is that they do not exhibit intracortical bone remodeling. As a consequence, age-related cortical bone loss is limited to resorption occurring on the endocortical bone surface. Bone loss associated with aging at this site is slow and compensated for by addition of bone onto the periosteal surface (Turner, et al. 2013a).

As in adult humans, cancellous bone remodeling continues throughout adult life in skeletally mature rats (Iwaniec and Turner 2013). An imbalance between bone formation and bone resorption is responsible for persistent bone loss during aging in both species. The trend for age-related cancellous bone loss observed in femur and lumbar vertebra in the present study is consistent with previous results where significant bone loss is noted between 8 months and 2 years of age (Turner et al. 2013a; Turner, et al. 2001). Caloric restriction accelerates age-related cancellous bone loss (Bodnar, et al. 2012; Mardon, et al. 2008a; Mardon, et al. 2008b), a finding consistent with the hypothesis that a decrease in leptin levels in the context of low energy availability contributes to bone loss. Leptin treatment, in spite of drastically

reducing body weight in growing *ob/ob* mice, results in increased longitudinal bone growth, increased bone formation, increased bone resorption, a net increase in bone mass and normalization of bone microarchitecture (Bartell et al. 2011; Hamrick, et al. 2005; Iwaniec, et al. 2007; Turner et al. 2013b).

In summary, hypothalamic leptin gene therapy, in spite of inducing weight loss, had minimal effects on bone mass, density, microarchitecture or biochemical markers of bone turnover. The lack of an effect of rAAV-Leptin gene therapy on bone in skeletally mature rats contrasts with the negative skeletal effects associated with similar weight loss induced by caloric restriction (Turner and Iwaniec 2011). There has been remarkable recent progress in gene therapy, including treatment of diseases of the CNS (Kantor, et al. 2014; Leone, et al. 2012). Our findings suggest that interventions, including hypothalamic leptin gene therapy, targeted toward increasing hypothalamic leptin levels have the potential to overcome leptin resistance and lower body weight without negatively impacting the skeleton.

Acknowledgments

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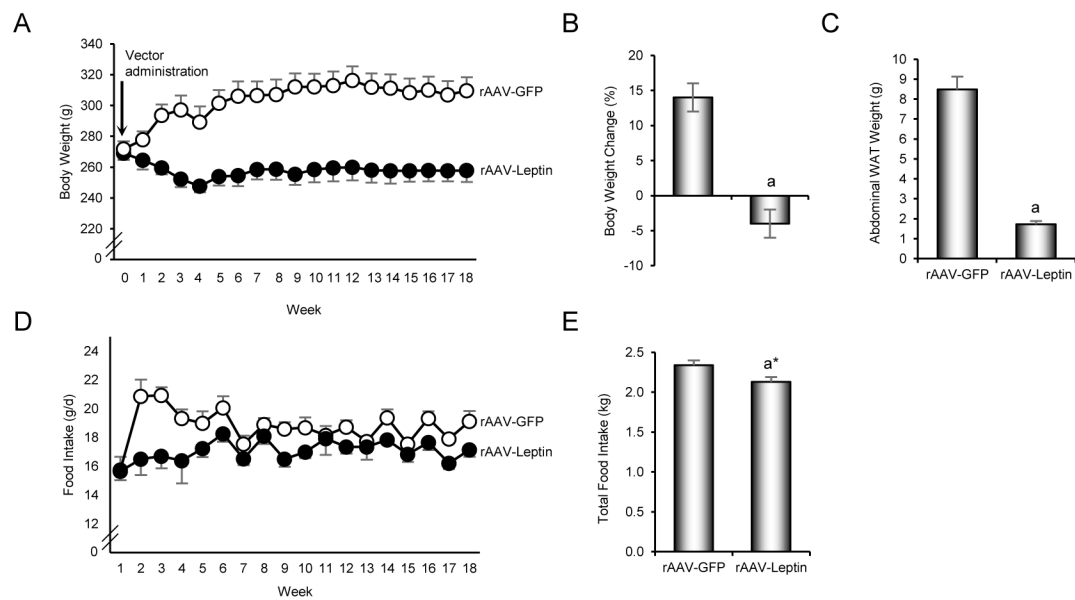
Figure 1.
Effects of increased hypothalamic leptin via rAAV-Leptin therapy on body weight over time (A), body weight change (B), abdominal white adipose tissue (WAT) weight (C), food intake over time (D), and cumulative food intake (E). Values are mean ± SE, n=7–10/group. [a]Different from rAAV-GFP, p   0.05. [a*]Different from rAAV-GFP, p < 0.1.
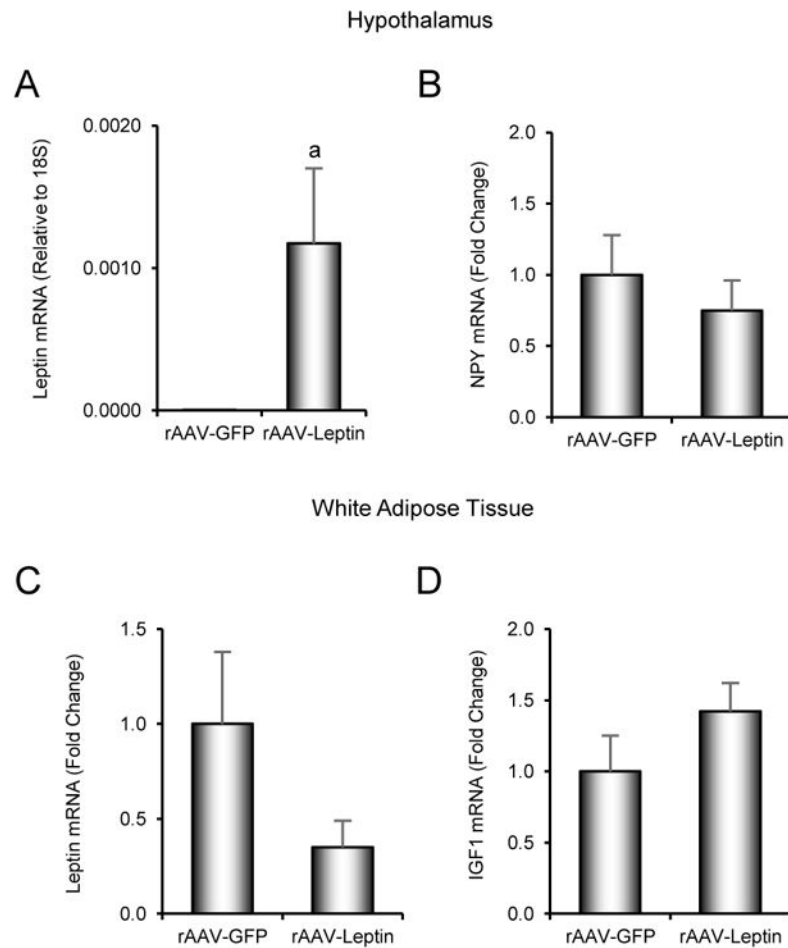
Figure 2.
Effects of increased hypothalamic leptin via rAAV-Leptin therapy on leptin (A) and NPY (B) mRNA expression in hypothalamus, and leptin (C) and IGF1 (D) mRNA expression in abdominal white adipose tissue. Values are mean ± SE, n=7–10/group. [a]Different from rAAV-GFP, p   0.05.
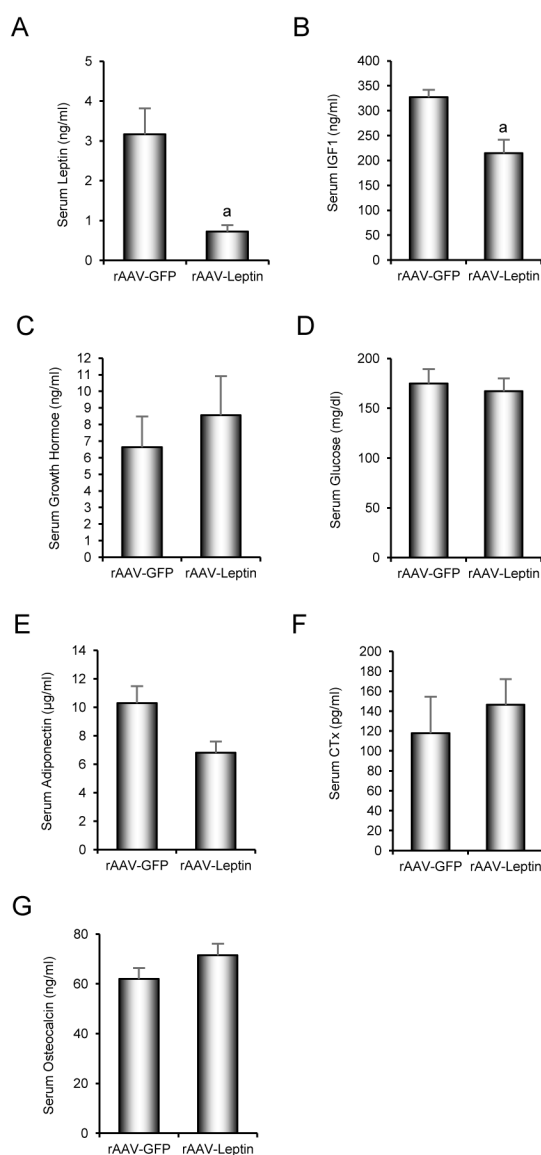
Figure 3.
Effects of increased hypothalamic leptin via rAAV-Leptin therapy on serum leptin (A), IGF1
(B), growth hormone (C), glucose (D), adiponectin (E), CTx (F), and osteocalcin (G). Values
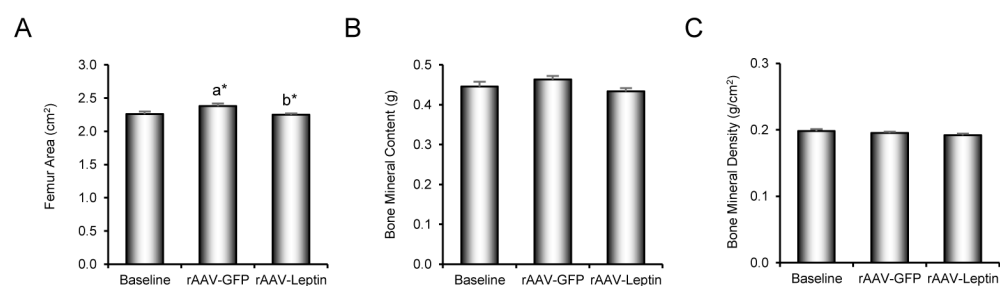are mean ± SE, n=7–10/group. [a]Different from rAAV-GFP, p ≤ 0.05.

Figure 4.
Effects of increased hypothalamic leptin via rAAV-Leptin therapy on femur area (A), bone mineral content (B), and bone mineral density (C). Values are mean ± SE, n=7–10/group. [a*]Different from Baseline, $p < 0.1$. [b*]Different from rAAV-GFP, $p < 0.1$.

Table 1

Effects of hypothalamic rAAV-Leptin therapy on cortical bone architecture in the femoral diaphysis and cancellous bone volume fraction (bone volume/tissue volume) and cancellous bone architecture in distal femur metaphysis and epiphysis.

Endpoint	Baseline	rAAV-GFP	rAAV-Leptin	FDR-adjusted p-value
Femur Diaphysis (cortical bone)				
Cross-sectional volume (mm^3)	3.09 ± 0.08	3.29 ± 0.06	$3.05 \pm 0.06^{b*}$	0.072
Cortical volume (mm^3)	2.01 ± 0.04	2.16 ± 0.02^{a}	$2.05 \pm 0.04^{b*}$	0.056
Marrow volume (mm^3)	1.08 ± 0.04	1.12 ± 0.04	1.00 ± 0.04	0.182
Cortical thickness (μm)	724 ± 8	753 ± 7	748 ± 13	0.135
I_{polar} (mm)	14.60 ± 0.73	$16.65 \pm 0.49^{a*}$	$14.46 \pm 0.58^{b*}$	0.071
Distal Femur Metaphysis (cancellous bone)				
Bone volume/tissue volume (%)	28.0 ± 3.4	$18.1 \pm 2.3^{a*}$	20.9 ± 1.5	0.072
Connectivity density ($1/mm^3$)	92.9 ± 5.6	59.3 ± 7.4^{a}	$70.6 \pm 5.6^{a*}$	0.052
Trabecular number (1/mm)	4.6 ± 0.2	$3.8 \pm 0.2^{a*}$	$4.0 \pm 0.1^{a*}$	0.058
Trabecular thickness (μm)	78 ± 4	69 ± 3	70 ± 2	0.195
Trabecular spacing (μm)	214 ± 11	$263 \pm 15^{a*}$	$245 \pm 8^{a*}$	0.087
Distal Femur Epiphysis (cancellous bone)				
Bone volume/tissue volume (%)	42.4 ± 1.0	$37.1 \pm 1.3^{a*}$	38.3 ± 0.7^{a}	0.056
Connectivity density ($1/mm^3$)	39.0 ± 2.1	35.9 ± 1.5	34.8 ± 2.0	0.378
Trabecular number (1/mm)	3.9 ± 0.1	3.7 ± 0.1	3.7 ± 0.1	0.364
Trabecular thickness (μm)	108 ± 1	102 ± 2	102 ± 2	0.108
Trabecular spacing (μm)	241 ± 9	256 ± 5	257 ± 8	0.328

Data are mean ± SE; n = 7 – 10/group

[a] Different from Baseline, p   0.05.

[a*] Different from Baseline, p < 0.1

[b*] Different from rAAV-GFP, p < 0.1

Table 2

Effects of hypothalamic rAAV-Leptin therapy on cancellous bone volume fraction (bone volume/tissue volume) and cancellous bone architecture in lumbar vertebra.

Endpoint	Baseline	rAAV-GFP	rAAV-Leptin	FDR-adjusted p-value
Lumbar Vertebra (cancellous bone)				
Bone volume/tissue volume (%)	36.5 ± 1.9	$28.9 \pm 2.05^{a*}$	32.7 ± 1.8	0.087
Connectivity density (1/mm^3)	50.4 ± 2.7	52.1 ± 3.1	56.0 ± 4.8	0.625
Trabecular number (1/mm)	4.1 ± 0.1	$3.6 \pm 0.13^{a*}$	3.9 ± 0.1	0.090
Trabecular thickness (μm)	87 ± 2	79 ± 2	82 ± 3	0.182
Trabecular spacing (μm)	236 ± 9	$273 \pm 11^{a*}$	252 ± 8	0.087

Data are mean ± SE; n = 7 – 10/group

[a*]Different from Baseline, p < 0.1