Increased Leptin Expression in the Dorsal Vagal Complex Suppresses Adiposity without Affecting Energy Intake and Metabolic Hormones

Stéphane Boghossian, Anne Lecklin,* Michael G. Dube,† Pushpa S. Kalra,† and Satya P. Kalra**

Abstract

BOGHOSSIAN, STÉPHANE, ANNE LECKLIN, MICHAEL G. DUBE, PUSHPA S. KALRA, AND SATYA P. KALRA. Increased leptin expression in the dorsal vagal complex suppresses adiposity without affecting energy intake and metabolic hormones. *Obesity.* 2006;14: 1003–1009.

Objective: Increased leptin transgene expression locally in hypothalamic sites suppresses weight and energy intake, enhances thermogenic energy expenditure, and differentially modulates metabolic hormones for an extended period. We evaluated whether a similar localized expression of leptin transgene in the dorsal vagal complex (DVC) in the caudal brain stem that also displays the biologically relevant leptin receptor would reproduce these varied responses and thus demonstrate functional connectivity between the hypothalamus and DVC.

Research Methods and Procedures: Adult female rats were microinjected with a recombinant adeno-associated virus encoding either rat leptin or green fluorescent protein gene (control) in the DVC. Food intake and body weight were monitored weekly, and metabolic variables were analyzed at the end of 10 weeks.

Results and Discussion: Increased leptin transgene expression in the DVC suppressed the time-related increase in body weight accompanied by a transient decrease in food intake at week 1 post-injection and little effect on thermogenic energy expenditure. That suppression of weight was due to decreased adiposity is shown by the markedly suppressed white adipose tissue-derived hormones, leptin and adiponectin. Circulating concentrations of pancreatic insulin, gastric ghrelin, and glucose levels were unchanged. This segregation of the varied effects of leptin expression in hypothalamic sites vs. DVC endorses the view that among the various endocrine organs under sympathetic nervous system control, only those leptin-activated neural circuits in the hypothalamus that suppress weight and adiposity on a long-term basis transverse through DVC en route to white adipose tissue.

Key words: autonomic pathways, gene therapy, hypothalamus, insulin, ghrelin

Introduction

Leptin, a product of the *ob* gene, plays an important role in the regulation and coordination of feeding behavior, energy expenditure, and metabolism (1–4). The biologically active long form of leptin receptor (OB-Rb) has been localized in multiple sites in the hypothalamus and caudal brain stem, the neuroaxis mediating integration of energy homeostasis by leptin (1–7). However, it is not known whether one or more than one neural pathway in this neuroaxis transmit leptin signaling to mediate the diverse hypothalamic effects of leptin. A single intracerebroventricular (ICV)[1] or intraparenchymal injection of leptin rostrally in the hypothalamic paraventricular nucleus (PVN), ventromedial nucleus (VMN), dorsomedial hypothalamus, and arcuate nucleus (ARC), and caudally in the dorsal vagal

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Departments of *Neuroscience and †Physiology and Functional Genomics, University of Florida McKnight Brain Institute, Gainesville, Florida.
Address correspondence to Satya P. Kalra, Department of Neuroscience, University of Florida McKnight Brain Institute, P.O. Box 100244, Gainesville, FL 32610-0244.
E-mail: skalra@mbi.ufl.edu

[1] Nonstandard abbreviations: ICV, intracerebroventricular; PVN, paraventricular nucleus; VMN, ventromedial nucleus; ARC, arcuate nucleus; DVC, dorsal vagal complex; BW, body weight; FI, food intake; MPOA, medial preoptic area; ARN, appetite-regulating network; BAT, brown adipose tissue; WAT, white adipose tissue; SNS, sympathetic nervous system; rAAV, recombinant adeno-associated virus; rAAV-lep, rAAV encoding rat leptin; rAAV-GFP, rAAV encoding green fluorescent protein gene; UCP-1, uncoupling protein-1.

complex (DVC) of the brain stem acutely inhibited feeding and body weight (BW) (1,2,8,9). Intraventricular infusion of leptin for 2 to 3 weeks, on the other hand, attenuated feeding transiently despite suppression of BW that lasted for the duration of the experiment (10–12). This temporal divergence of the two responses in conjunction with a sustained increase in energy expenditure implied the existence of distinct leptin-sensing pathways and/or mechanisms that integrate energy intake and expenditure. Indeed, subsequent results obtained by leptin gene transfer into hypothalamic sites for stable, long-term increase in leptin supply endorsed the existence of separate pathways (13–19). Enhanced leptin transgene expression in the medial preoptic area (MPOA) suppressed BW gain without reducing food intake (FI) or modulating the appetite-regulating network (ARN) in the hypothalamus, but it enhanced the non-shivering brown adipose tissue (BAT)-mediated thermogenesis (14,15). On the other hand, similar selective leptin transgene expression in the PVN, VMN, and ARC suppressed BW and FI and modified operation of the ARN together with increased BAT thermogenesis (14,15). Enhanced leptin expression in these forebrain sites modulated various circulating metabolic hormones implicated in weight homeostasis in diverse manners (13–24). Although blood levels of white adipose tissue (WAT)-derived leptin and adiponectin and pancreatic insulin were markedly suppressed, gastric ghrelin levels were elevated (13–24). Blood glucose levels were also significantly suppressed in these rodents (14–23).

The leptin-sensing hypothalamic mechanisms that confer weight homeostasis and regulate hormonal secretions are hypothesized to engage descending neural circuits that innervate endocrine glands in the periphery. Retrograde tract tracing studies showed that sympathetic nervous system (SNS) outflow from various hypothalamic sites is likely to traverse caudally through the brain stem en route to WAT, BAT, the pancreas, and the stomach (25–33). However, the precise route of SNS efferents from the hypothalamus to these peripheral endocrine organs is unknown. On the basis of cumulative evidence from gene therapy (13–19) and tract-tracing investigations (25–33), we hypothesize that distinct leptin-sensing SNS pathways descend caudally from the hypothalamus through the brain stem en route to peripheral target organs. The aim of the current investigation, therefore, was to assess the long-term impact of enhanced leptin transgene expression in the DVC of the brain stem on FI, BW, energy expenditure, circulating levels of the WAT-derived leptin and adiponectin, pancreatic insulin, gastric ghrelin, and glucose levels.

Research Methods and Procedures

Animals

Adult female Sprague-Dawley rats weighing 240 to 250 grams (Harlan Sprague Dawley, Inc., Indianapolis, IN) were housed individually in a light- (lights on 5 AM to 7 PM) and temperature-controlled room under specific pathogen-free conditions. Standard rat chow (LM-485; 3.4 kcal/g; Teklad, Madison, WI) and water were available ad libitum. The animal protocols were approved by the Institutional Animal Care and Use Committee.

Microinjection of Recombinant Adeno-Associated Virus (rAAV) Encoding Rat Leptin (rAAV-lep) in the DVC

Rats were anesthetized with ketamine/xylazine (100 mg/kg BW ketamine + 15 mg/kg BW xylazine) and were microinjected bilaterally with a non-immunogenic, non-pathogenic rAAV encoding the green fluorescent protein gene (rAAV-GFP; 9×10^7 particles, controls) or rAAV-lep (8×10^7 particles) into the DVC. The stereotaxic coordinates for DVC injections were: 4.8 mm from interaural line, 0.5 mm lateral to midline, and 5.0 mm below the dura (9). rAAV-lep or rAAV-GFP (1 μl) was slowly infused on each side over a 2-minute period, and the injector was removed 5 minutes later (14,16). The vectors used in this study were packaged, purified, concentrated, and titered as previously described (13–19). Briefly, the vector pTR-CBA-Ob EcoRI fragment of pCR-rOb (a gift from Dr. Roger H. Unger, Southwestern Medical School, Dallas, TX) containing rat leptin cDNA was subcloned into rAAV vector plasmid pAAVβGEnh after deleting the EcoRI fragment carrying the β-glucuronidase cDNA sequence (13,34,35). The control vector, rAAV-GFP, was similarly constructed to encode the *GFP* gene (13,35).

Experimental Design

FI and BW were monitored on a weekly basis for 10 weeks after DVC injections. At the end of the experiment, animals were sacrificed by decapitation from 9–11 AM. Blood was collected from the trunk, and serum was frozen for analysis of metabolic hormones. The DVC and the hypothalamus were dissected out for subsequent analyses of leptin mRNA expression by reverse transcriptase polymerase chain reaction, and BAT was dissected out for uncoupling protein-1 (UCP-1) mRNA expression by dot-blot hybridization (13–19).

Analyses

Leptin mRNA Expression in the DVC. Total RNA was extracted from the DVC and the hypothalamus using an RNA isolation kit (Qiagen, Inc., Valencia, CA) as described (13–19). First strand cDNA was obtained using an RNA polymerase chain reaction kit (reverse transcription system; Promega, Madison, WI). Primers were designed to the rat leptin gene to encompass a 308-base-pair region of the coding sequence (36) (Gene Bank accession no. D49653): sense, 3′-CCC ATT CTG AGT TTG TCC; and antisense, 3′-GCA TTC AGG GCT AAG GTC. Cyclophilin, used as endogenous control, was generated as a 470-base-pair prod-

uct (Gene Bank accession no. M19533): sense, 3′-GAC AAA GTT CCA AAG ACA GCA GAA A; and antisense, 3′-CTG AGC TAC AGA AGG AAT GGT TTG A.

UCP-1 mRNA Expression in the BAT. UCP-1 mRNA expression in BAT was measured as described earlier (13–15). In brief, the total RNA was isolated from the BAT using an RNA isolation kit (STAT-60; Teltest, Inc., Friendswood, TX), and a dot-blot hybridization analysis of UCP-1 mRNA levels was performed (13–15).

Metabolic Hormones. Serum leptin, adiponectin, ghrelin, and insulin were measured by RIA kits (Linco Research, St. Charles, MO) as previously described (13–15). Serum glucose levels were measured with a glucose meter (Glucometer Elite XL; Bayer, Elkhart, IN).

Statistical Analyses

BW and FI were analyzed using either two-way ANOVA or Student's *t* test, as appropriate. Leptin, insulin, adiponectin, glucose, leptin mRNA levels, and UCP-1 mRNA levels were analyzed using one-way ANOVA followed by post hoc 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 DVC rAAV-lep Microinjection on Leptin mRNA Expression in the DVC and UCP-1 mRNA Expression in the BAT

At 10 weeks post-injection, there was a 3-fold increase in leptin mRNA expression in brain tissue containing the DVC from rAAV-lep as compared with rAAV-GFP-injected rats ($p < 0.05$, Figure 1A). Leptin mRNA expression was unchanged in the rostral hypothalamic fragment of rAAV-lep-injected rats. The increase in leptin gene expression in the DVC failed to affect UCP-1 mRNA expression in the BAT (Figure 1B), thereby suggesting little impact on non-shivering thermogenic energy expenditure in these rats.

Effects of DVC rAAV-lep Microinjection on BW and FI

Figure 1C shows the time course of changes in BW in response to rAAV-GFP or rAAV-lep microinjections in the DVC. rAAV-GFP-injected rats steadily gained weight to attain 14% increase from initial values at the end of 10 weeks ($p < 0.05$). Increased leptin transgene expression in the DVC completely blocked this time-related BW gain. A significant decrease in weight gain from the control group was first evident at Week 5 ($p < 0.05$) and was maintained through the remainder of the duration of the experiment.

The time course of FI in response to rAAV-lep treatment was markedly different from that of BW response. FI decreased significantly initially at Week 1 post-injection ($p < 0.05$) but rapidly returned to the control range by Week 2 and was maintained, thereafter, within the control range (Figure 1D).

Effects of DVC rAAV-lep Microinjection on Circulating Metabolic Hormones

Increased leptin transgene expression in the DVC suppressed both adipocyte-derived hormones, leptin and adiponectin (Figure 2, A and B). Serum leptin and adiponectin levels in these rats were reduced by 50% and 19%, respectively, as compared with those found in rAAV-GFP-treated rats ($p < 0.05$). On the other hand, serum insulin, glucose, and ghrelin levels were unaffected by DVC rAAV-lep microinjection (Figure 2, C–E).

Discussion

The current study extends our continuing attempts to identify the route of functional efferent pathways mediating the hypothalamic effects of leptin on appetite, BW, adiposity, energy expenditure, and metabolic variables with the aid of gene transfer technology that enhances ectopic leptin expression in Ob-Rb-expressing neural sites (1–7,13,14, 16,37–39). There are several new findings of this study. First, the results show that augmented leptin action, as reflected by enhanced leptin mRNA expression selectively in the DVC, blocked the gradual time-related increase in BW. Furthermore, as was the case in the hypothalamus (13–19,21,40), there was no evidence of resistance to this restraint on BW exerted by the sustained leptin action. It is generally believed that the brain stem may modulate short-term intrameal energy consumption. In this context, our findings are the first to demonstrate the existence of neural elements in the brain stem that can impose long-term control on energy balance. Second, we observed detectable levels of leptin mRNA expression in the brain stem to suggest that, as in the case with the hypothalamus (19), leptin may be elaborated locally in the brain stem.

Third, maintenance of weight at the pre-injection range for extended periods is likely a result of decreased body fat content because circulating levels of leptin and adiponectin, the WAT-derived hormones shown previously by us and others to quantitatively correlate with body fat depot (1–4,24), were markedly suppressed. Indeed, central rAAV-lep therapy markedly suppresses episodic leptin secretion contemporaneous with fat depletion (23). Similar long-term suppression of fat accumulation along with lowered serum leptin and adiponectin levels without any adverse effects on lean muscle mass was observed in rats and mice receiving either a single ICV injection or microinjection of rAAV-lep into the PVN, VMN, ARC, and MPOA in the forebrain or receiving continued peripheral and central leptin infusions (10–19). Our current results also extend earlier reports of a decrease in BW at 24 to 48 hours after either ICV injection or microinjection of leptin into selected forebrain sites and the DVC. However, unlike those in the current study, the effects on leptin and adiponectin levels to determine effects on adiposity were not assessed in these investigations (1,8,9).

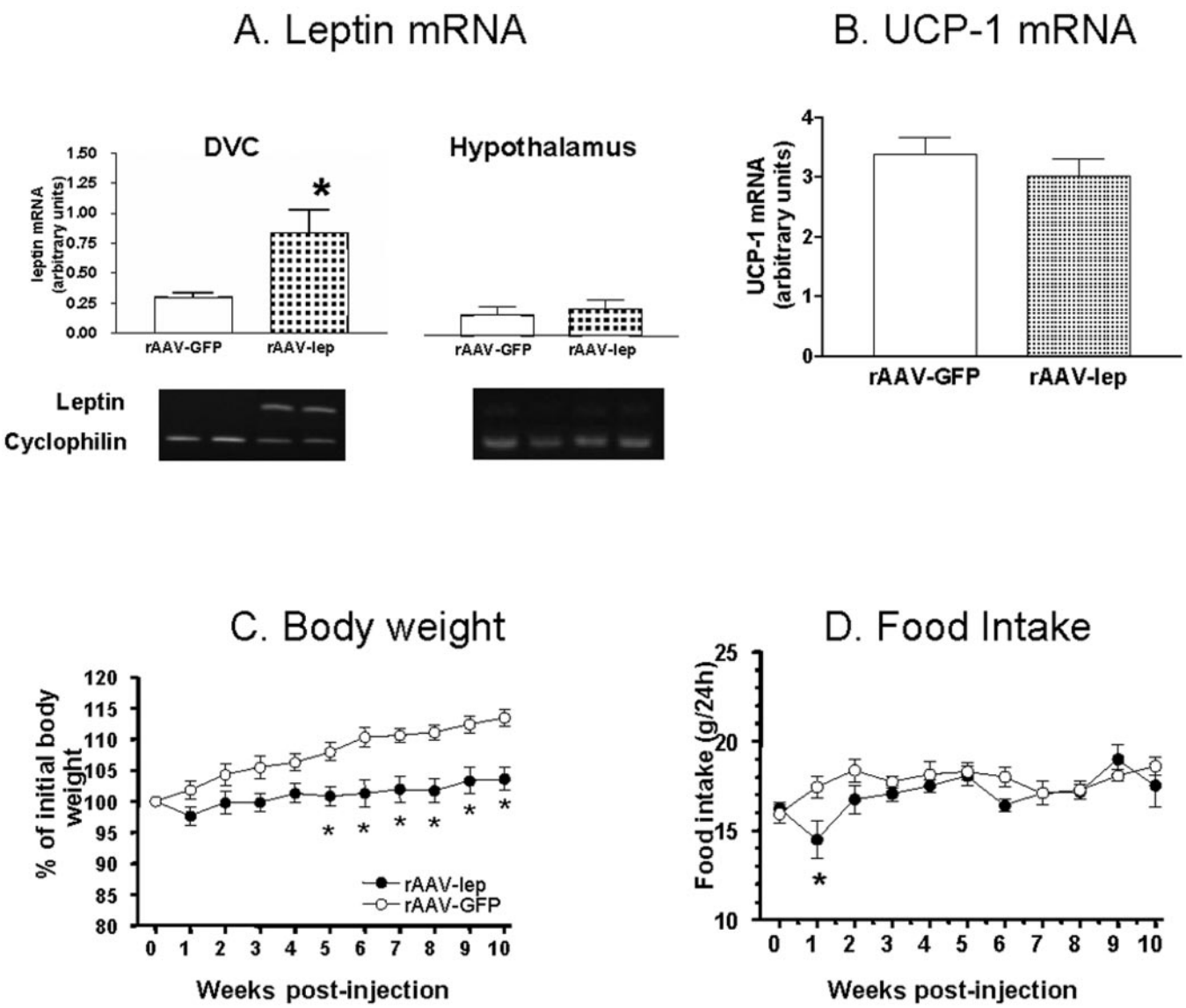

Figure 1: (A) Leptin mRNA expression, as analyzed by reverse transcriptase polymerase chain reaction, in neural tissue containing DVC or hypothalamus of rAAV-GFP-injected (control) and rAAV-lep-injected rats. * $p < 0.05$ vs. control. (B) The effects of DVC injection of either rAAV-GFP (control) or rAAV-lep on UCP-1 mRNA expression in BAT. (C and D) BW and FI profiles of DVC rAAV-GFP and rAAV-lep-injected rats during the 10-week course of experiment. * $p < 0.05$ vs. control at that time interval.

Fourth, evaluation of the long-term effects of stably expressed leptin transgene in the current study reveals a temporal divergence in leptin-induced restraint on energy consumption and weight gain. In previous studies, suppression of FI accompanied the loss of BW at 24 to 48 hours after microinjection of leptin in the DVC (9). In contrast, we observed that after a single rAAV-lep microinjection in the DVC, FI decreased at Week 1 post-injection but returned rapidly to the control range despite a complete arrest of the time-related weight gain. Although unexpected, this dissociation of FI and BW responses is not unprecedented. Similar divergences of FI and BW responses have been reported in response to either rAAV-lep microinjection in the MPOA or ICV injection of extremely low doses of rAAV-lep that do not affect hypothalamic ARN (13,14). Continuous infusion of leptin itself either peripherally or centrally in rodents also elicited a similar temporal dissociation of the two responses (10–12). It is possible that dissipation of leptin restraint on FI is a consequence of development of leptin resistance by central targets mediating energy intake but not weight homeostasis (1–5,12,41). However, the observation that either a single ICV injection or microinjection of rAAV-lep in the PVN, VMN, or ARC suppressed FI and age-related weight gain for long periods is not consistent

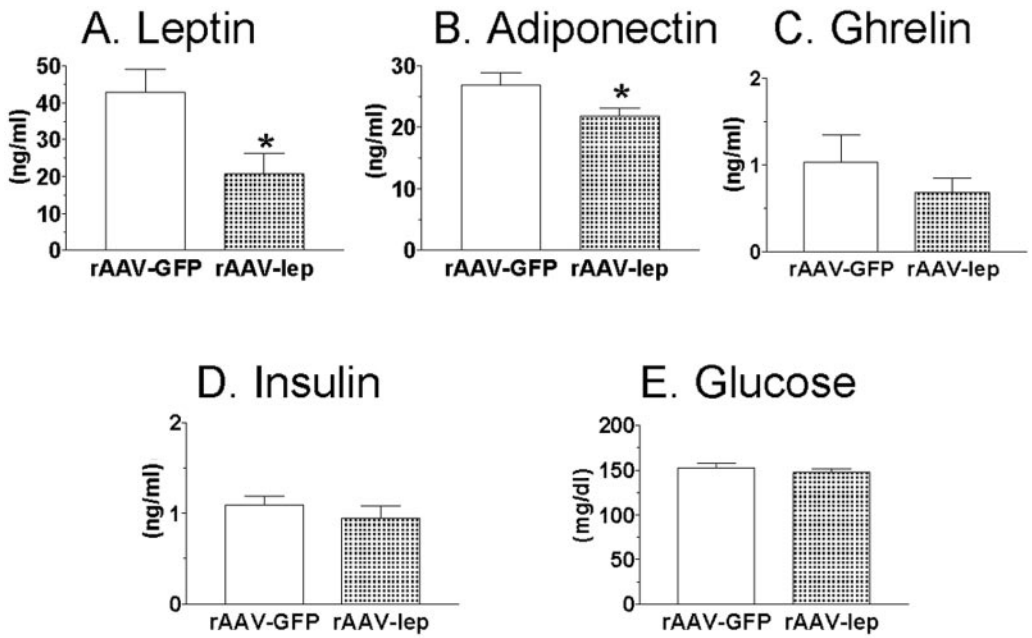

Figure 2: Circulating levels of leptin (A), adiponectin (B), ghrelin (C), insulin (D), and glucose (E) at 10 weeks post-injection of rAAV-GFP or rAAV-lep in the DVC. * $p < 0.05$ vs. rAAV-GFP.

with the notion of development of resistance to leptin by central target elements (13–19,42,43). On the contrary, the temporal divergence of the effects on energy consumption and weight gain seen in MPOA and DVC rAAV-lep microinjected rats are in accord with an alternate possibility. We propose that the observed SNS link between the MPOA and DVC (25–33) is engaged primarily in relay of long-term leptin restraint on weight gain due to fat deposition and not due to energy consumption. If this is the case, then the neural and cellular mechanisms responsible for suppression of the time-related weight gain concomitant with normal daily intake remain to be ascertained. Likewise, it is of interest to note that the failure of rAAV-lep microinjection in the MPOA to inhibit FI was attributed to the ineffectiveness of the localized leptin transgene expression to affect the caudally located ARN (1,14–16). In previous studies, it was found that leptin is undetectable in the hypothalamus and in the periphery after intraventricular injection of rAAV-lep (16,19,43). Thus, the current results do not rule out a similar lack of modulation of hypothalamic ARN by transgene expression in the DVC.

Tract-tracing studies have documented the existence of anatomical pathways that relay sympathetic outflow from hypothalamus to BAT, pancreas, and stomach (1–5,25–33,44). In this context, the fifth new finding relates to the lack of leptin-induced sympathetic outflow through the DVC to these endocrine organs. Although leptin transgene expression in the hypothalamic MPOA, VMN, PVN, and ARC increased BAT UCP-1 mRNA expression, a similar increase in leptin expression in the DVC was ineffective. One can infer that unlike that seen in forebrain sites, increased thermogenesis played little role in maintaining reduced adiposity in DVC rAAV-lep-treated rats. Additionally, leptin has been shown to promote increased general activity, fat oxidation, and adipocyte apoptosis (1–5,45,46). Thus, it is likely that increases in the magnitude of these responses reduced BW despite normal rates of thermogenic energy expenditure in these rats.

Increased leptin transgene expression in hypothalamic sites suppressed insulin secretion in rodents consuming normal rodent chow and blocked the high-fat-diet-induced hyperinsulinemia, accompanied by a modest reduction in blood glucose levels (13–19,23,34). On the other hand, we have now observed that increased leptin transgene expression in the DVC failed to decrease blood insulin and glucose levels. Similarly, serum ghrelin levels were unchanged in these rats, in contrast to the marked elevations elicited by leptin transgene expression in hypothalamic sites (13–19,23).

In conclusion, these results demonstrate the long-term efficacy of DVC leptin transgene expression in curbing the time-related increase in weight and fat deposition; however, it causes only a transient suppression of FI and no effect on circulating insulin, ghrelin and glucose levels, and thermogenic energy expenditure. This long-term segregation of effects of DVC leptin transgene expression from those observed after similar hypothalamic leptin transgene expression is novel and consistent with the proposal that efferent relay of SNS outflow propagated by leptin from hypothalamic target

sites bypasses DVC en route to peripheral SNS targets, namely BAT, the pancreas, and the stomach.

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