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Short Article

Endogenous Leptin Signaling in the Caudal Nucleus Tractus Solitarius and Area Postrema Is Required for Energy Balance Regulation

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SUMMARY

Medial nucleus tractus solitarius (mNTS) neurons express leptin receptors (LepRs), and intra-mNTS delivery of leptin reduces food intake and body weight. Here, the contribution of endogenous LepR signaling in mNTS neurons to energy balance control was examined. Knockdown of LepR in mNTS and area postrema (AP) neurons of rats (LepRKD) via adeno-associated virus short hairpin RNA-interference (AAV-shRNAi) resulted in significant hyperphagia for chow, high-fat, and sucrose diets, yielding increased body weight and adiposity. The chronic hyperphagia of mNTS/AP LepRKD rats is likely mediated by a reduction in leptin potentiation of gastrointestinal satiation signaling, as LepRKD rats showed decreased sensitivity to the intake-reducing effects of cholecystokinin. LepRKD rats showed increased basal AMP-kinase activity in mNTS/AP micropunches, and pharmacological data suggest that this increase provides a likely mechanism for their chronic hyperphagia. Overall these findings demonstrate that LepRs in mNTS and AP neurons are required for normal energy balance control.

INTRODUCTION

Fifteen years after the discovery of leptin (Zhang et al., 1994), the adipose tissue-derived hormone continues to influence thinking about the nature of central nervous system (CNS) circuits that control energy balance. Explanations for the potent decrease in food intake and increase in energy expenditure triggered by central leptin signaling focus almost exclusively on one region of leptin receptor (LepR)-bearing nuclei, the arcuate (ARC) hypothalamus (Barsh and Schwartz, 2002). Only recently has attention been directed toward evaluating contributions of other, extra-ARC and extra-hypothalamic LepRs (Dhillon et al., 2006; Fulton et al., 2006; Hommel et al., 2006; Myers et al., 2009). Two recent reports are beginning to move the attention of the field away from the ARC-centric notion of leptin action by showing that in the absence of LepR signaling in the neurons of the hypothalamic ventromedial nucleus (VMN) or the midbrain ventral tegmental area (VTA), rodents become hyperphagic (Dhillon et al., 2006; Hommel et al., 2006). These data contribute to a developing perspective that the energy balance effects of leptin are anatomically *distributed* rather than centered in the ARC (Grill and Hayes, 2009; Hommel et al., 2006; Huo et al., 2007; Myers et al., 2009).

Behavioral analyses of meal patterns show that the intake-reducing effects following leptin treatment occur by a reduction in meal size, not by an alteration in meal number or frequency (Eckel et al., 1998; Kahler et al., 1998). Interestingly, the same meal pattern effect is produced by within-meal satiation signals arising from the gastrointestinal (GI) tract (Moran, 2006; Ruttimann et al., 2009; West et al., 1984). The common meal size suppressive effects of leptin and GI satiation signals suggest that the intake-reducing effect of leptin may result from interactions with the CNS processing of GI satiation signals (e.g., gastric distension, glucagon-like-peptide-1, and cholecystokinin [CCK]) (Huo et al., 2007; Matson et al., 2000; Schwartz and Moran, 2002; Williams et al., 2006). Data supporting the hypothesis that neurons of the nucleus tractus solitarius (NTS) serve as a site of leptin and GI satiation signal convergence show that: (1) CNS leptin delivery amplifies the neurophysiologic response of NTS neurons to gastric distension (Schwartz and Moran, 2002), (2) 39% of mNTS neurons are responsive to leptin and gastric distension (Huo et al., 2007), and (3) a subthreshold dose of hindbrain-delivered leptin amplifies the intake-reducing effects of a subthreshold volume of gastric distention (Huo et al., 2007). Collectively these findings indicate that mNTS LepR signaling can reduce food intake by interacting with GI-vagally transmitted satiation signals. Whether mNTS LepR-expressing neurons are *required* for normal food intake control and overall energy balance regulation is unknown.

It is likely that a common intracellular signaling pathway(s) within mNTS neurons accounts for the interaction between leptin signaling and GI satiation signal processing. Examination of the intracellular pathways that may mediate the intake-suppressive effects of leptin action in the mNTS focus on the fuel-sensing enzyme, adenosine monophosphate-activated protein kinase

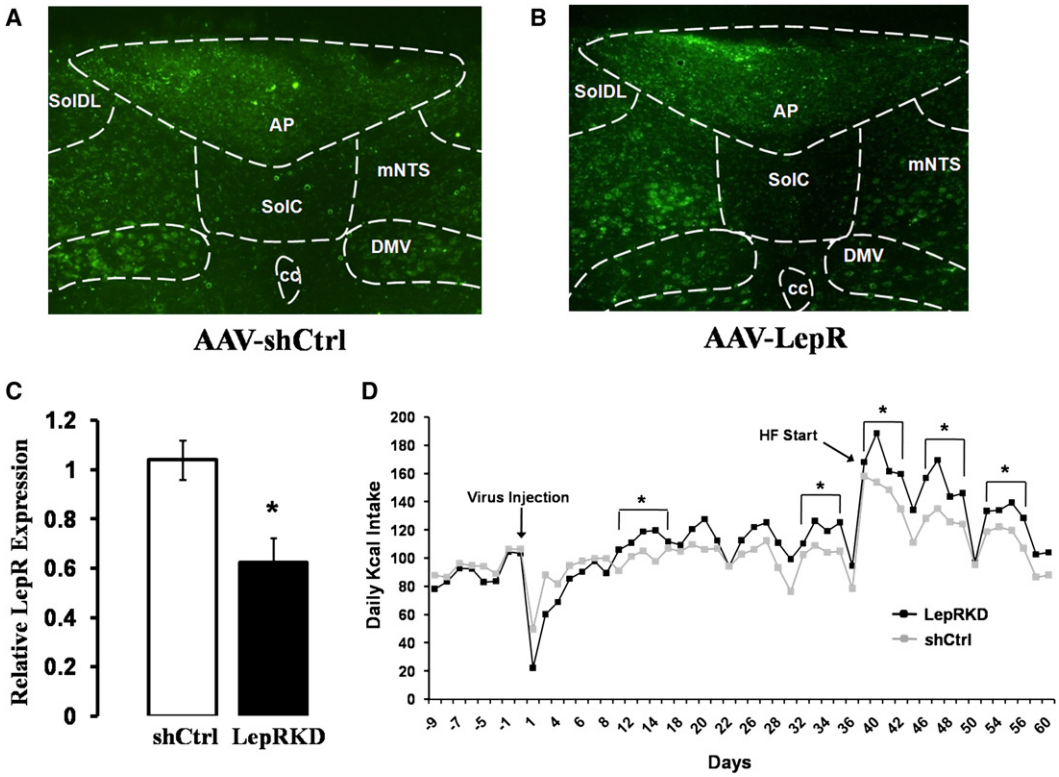

Figure 1. Leptin Receptor Knockdown in the mNTS and AP Increases Daily Caloric Intake

(A and B) Representative immunofluorescent analysis showing the extent of AAV-shCtrl (A) and AAV-LepR (B) spread, with EGFP-expressing neurons being identified in nuclei that contain the LepR: mNTS and AP. EGFP-expressing neurons are also found in non-LepR-expressing nuclei: DMV, dorsolateral NTS (SolDL), and solitary commissural (SolC). cc, central canal.

(C) Representative qPCR reveals significant suppression in LepR mRNA in mNTS/AP micropunched tissue for AAV-LepR-treated rats compared to AAV-shCtrl rats. *$p < 0.05$.

(D) Daily Kcal intake for LepRKD and shCtrl rats pre- and postviral delivery while maintained on chow or high fat (HF) diet (60% Kcal from fat). * = $p < 0.05$ for bracketed weekly averages.

(AMPK) (Hayes et al., 2009). The intake-reducing effects of hindbrain leptin delivery were found to be mediated by AMPK, as increased hindbrain AMPK activity by fourth intracerebroventricular (i.c.v.) AICAR delivery reversed hindbrain leptin-induced inhibition of food intake and AMPK activity in NTS-enriched lysates. These findings highlight a role for mNTS AMPK activity in energy balance control.

To assess the role of *endogenous* leptin signaling in mNTS LepR-expressing neurons to the normal physiology of energy balance control, we utilized RNAi-mediated LepR knockdown (Hommel et al., 2006). Rats received bilateral mNTS-parenchymal injections of an adeno-associated virus (AAV2) engineered to express either shRNA targeting LepR mRNA (AAV-shLepR) or scrambled shRNA (AAV-shCtrl). The daily caloric intake, energy expenditure parameters (core temperature and physical activity), body weight gain, and white adipose tissue (WAT) mass of rats maintained on chow or high-fat (HF) diet were compared. To determine whether endogenous mNTS leptin signaling contributes to the food intake-reducing effects of GI satiation signals, responses to intraperitoneal (i.p.) CCK were assessed. The effect of mNTS-directed AAV-shLepR on basal hindbrain AMPK activity and the feeding effect of pharmacological inhibition of hindbrain AMPK activity were also examined.

RESULTS

Quantification of In Vivo LepR Knockdown and Histological Confirmation of Viral Infection

Figures 1A and 1B illustrate representative histological confirmation of AAV-shCtrl and AAV-shLepR infection of mNTS and AP neurons by expression of enhanced green fluorescent protein (EGFP; coexpressed by the AAV). In addition, adjacent non-LepR-expressing nuclei of the dorsal motor nucleus of the vagus (DMV), dorsolateral NTS, solitary commissural, and lateral NTS were also infected by the AAV and express EGFP. However, given that LepRs are expressed only in the mNTS and AP in the infected region (Huo et al., 2007; Li et al., 1999), only RNA extracted from cells from micropunches of AAV-infected mNTS/AP tissue were analyzed by quantitative real-time polymerase chain reaction (qPCR). Representative qPCR analysis revealed a 41% decrease in LepR mRNA in mNTS/AP neurons infected with AAV-shLepR (n = 6), compared with neurons infected with AAV-shCtrl (n = 12; Figure 1C).

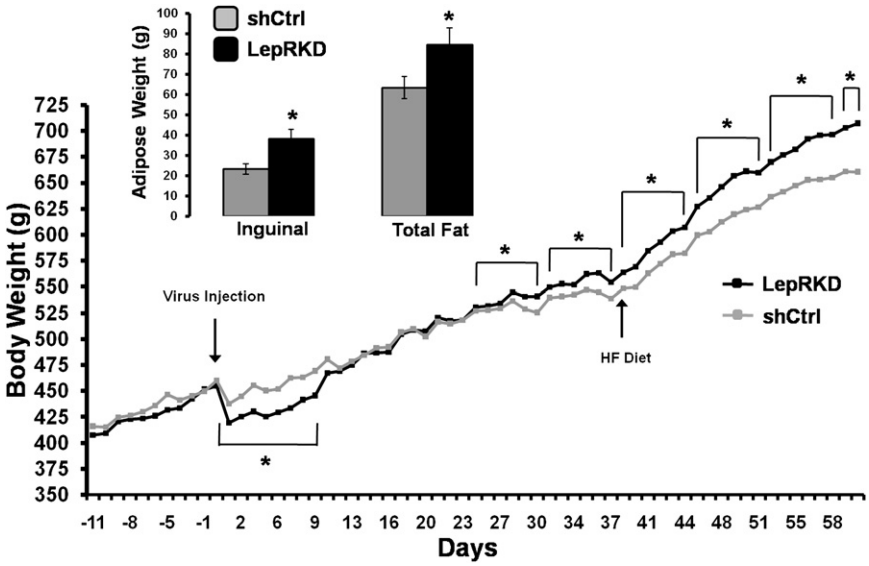

Figure 2. Increased Body Weight Gain Following Knockdown of mNTS/AP LepR

Cumulative body weight of chow-maintained LepRKD and shCtrl rats pre- and post-mNTS/AP-directed AAV delivery. *$p < 0.05$ for bracketed weekly averages. (Inset) Graph shows inguinal and total WAT mass for LepRKD and shCtrl rats. *$p < 0.05$.

Knockdown of mNTS and AP LepR Increases Food Intake and Body Weight Gain

LepRKD rats maintained on standard chow showed a significant increase in weekly averaged daily caloric intake during the second and fifth week postviral delivery compared to shCtrl rats (Figure 1D). When the diet was switched to HF diet at 5.5 weeks postviral delivery, weekly averaged daily caloric intake was significantly greater in LepRKD rats than shCtrl rats for the next 3 weeks.

LepRKD rats showed a significantly greater decrease in average body weight compared to shCtrl rats in the first 9 days following viral delivery, attributed to the weight loss seen within the first 24 hr following viral injection. However, beginning 3.5 weeks postviral delivery, body weight for LepRKD rats was significantly greater than that of shCtrl rats (Figure 2). This significant increase in body weight gain for LepRKD rats continued when rats were switched to HF diet for the remaining 3 weeks of the experiment. Postmortem analysis of WAT depots (8.5 weeks post-AAV delivery) revealed that the inguinal WAT mass was significantly greater in LepRKD rats compared to shCtrl rats (Figure 2, inset). There was no significant difference in retroperitoneal (19.7 ± 1.6 g versus 17.4 ± 1.4 g), epididymal (20.0 ± 1.9 g versus 16.7 ± 1.4 g), or perirenal (6.6 ± 0.8 g versus 5.8 ± 0.8 g) WAT depots between the LepRKD and shCtrl rats, respectively. The increase in inguinal fat mass accounted for the majority of the increase in total WAT mass for LepRKD rats compared to shCtrl rats (Figure 2, inset).

Analysis of core body temperature and physical activity for LepRKD and shCtrl rats showed no differences in either energy expenditure parameter for either group following AAV delivery compared to within-subject pre-AAV delivery baseline values (see Figures S1 and S2 available online).

Endogenous LepR Signaling in mNTS/AP Neurons Is Required for the Intake-Suppressive Effects of CCK

To test the hypothesis that the intake-suppressive effects of CNS LepR signaling result from potentiation of the intake-inhibitory effects of GI satiation signals within mNTS neurons, 15% sucrose intake of LepRKD and shCtrl rats was tested following i.p. injection of the GI satiation signal CCK. Figure 3A shows that for both groups, sucrose intake following vehicle injection was identical prior to viral delivery, as was the intake-suppressive effect of CCK (3 μg/kg). By contrast, 4 weeks following viral delivery, clear differences in sucrose intake were observed, with LepRKD rats consuming significantly more sucrose under vehicle-injection conditions compared to shCtrl rats (Figure 3A). This significant increase in sucrose intake occurred within 10 min of sucrose access (first meal) and persisted for the 1 hr duration of testing. LepRKD rats showed reduced sensitivity to the intake-suppressive effects of CCK (with no significant inhibition of sucrose intake) compared to shCtrl rats that continue to display a significant inhibition of sucrose intake by CCK.

To determine whether baseline hyperphagia of sucrose and reduced sensitivity to the intake-suppressive effects of CCK in LepRKD rats were selective to a palatable test diet, the effects of i.p. CCK (3 μg/kg) on maintenance chow was examined in other rats. Six weeks postviral delivery, CCK-treated LepRKD rats failed to significantly suppress 30 min chow intake, while the same treatment significantly suppressed chow intake in shCtrl rats (32.9% ± 22.7% versus 67.5% ± 19.5% suppression compared to within-subject intakes following i.p. saline, respectively).

Endogenous LepR Signaling in mNTS/AP Neurons Controls for Food Intake by an AMPK-Dependent Mechanism

The effect of mNTS-directed AAV-LepR on basal hindbrain AMPK activity (ad libitum chow-fed rats) was evaluated through immunoblot analysis of phosphorylation of AMPKα2 (pAMPKα2) and total AMPKα levels in mNTS/AP tissue lysates compared to pAMPKα2 levels in shCtrl rats 7 weeks post-AAV delivery. Figure 3B shows significant elevations of basal pAMPKα2 in LepRKD rats compared to shCtrl rats. Total AMPKα levels were equivalent.

To determine whether elevated pAMPKα2 levels in mNTS/AP of LepRKD rats contributes to their chronic hyperphagia, the feeding effect of fourth i.c.v. compound C delivery was tested in chow-maintained LepRKD and shCtrl rats 3 weeks postviral delivery. Figure 3C shows that daily (24 hr) chow intake for LepRKD rats was significantly greater than that of shCtrl rats following fourth i.c.v. vehicle administration. Compound C (15 μg), at a dose subthreshold for effect in shCtrl rats, significantly

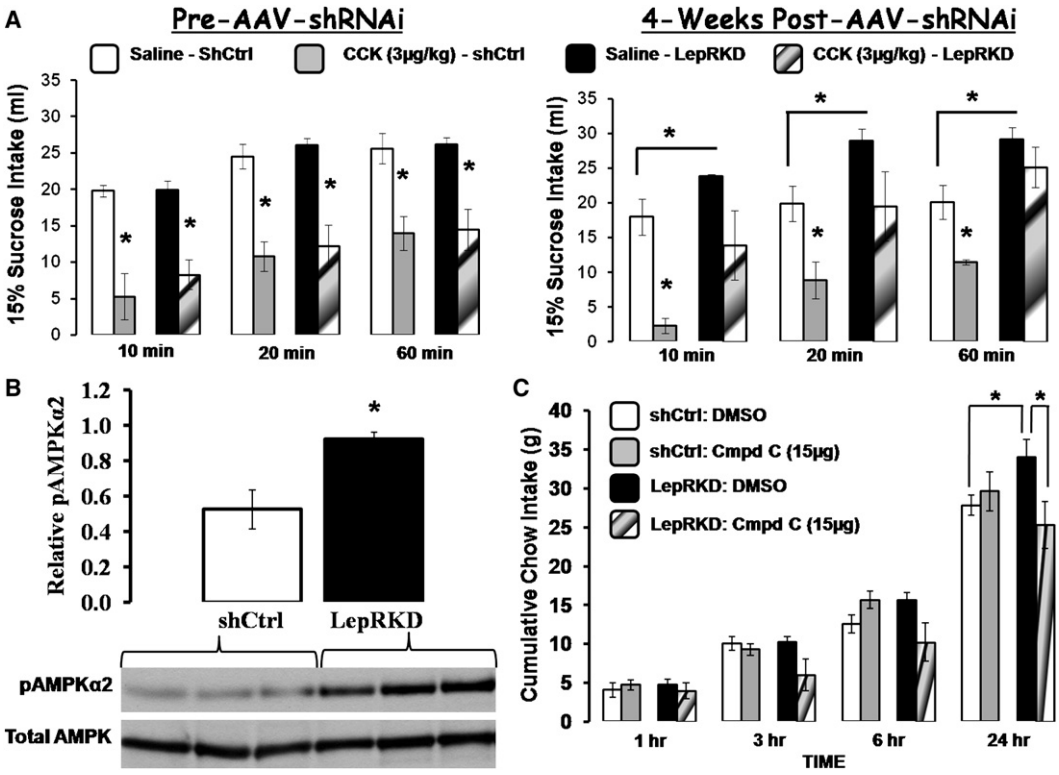

Figure 3. Endogenous LepR Signaling in mNTS/AP Neurons Is Required for the Intake-Suppressive Effects of CCK and Controls for Food Intake via an AMPK-Dependent Mechanism

(A) Cumulative 15% sucrose intake following i.p. injection of CCK (3 μg/kg) or vehicle (0.9% saline) for LepRKD and shCtrl rats pre- and 4 weeks post-mNTS-directed AAV delivery. *$p < 0.05$.

(B) LepRKD rats showed increased pAMPKα2 levels in mNTS/AP micropunched tissue compared to shCtrl rats under ad libitum-fed conditions. Representative immunoblots for total AMPK and pAMPKα2 are shown. Relative pAMPKα2 = the ratio of pAMPKα2 to total AMPK. *$p < 0.05$.

(C) Cumulative chow intake for LepRKD and shCtrl rats following fourth i.c.v. delivery of compound C (15μg) or vehicle (DMSO). *$p < 0.05$.

suppressed 24 hr food intake in LepRKD rats to a level equal to the intake of shCtrl rats following vehicle administration. There was a nonsignificant trend for inhibition of intake by compound C in LepRKD rats at 6 hr postinjection ($p = 0.11$).

DISCUSSION

Endogenous CNS leptin signaling plays a pivotal role in energy balance control (Bence et al., 2006; Cohen et al., 2001; Dhillon et al., 2006; Hommel et al., 2006; Myers et al., 2009; Zhang et al., 1994). Less is known, however, about which of the various nuclei and possible mediating mechanisms are relevant to the control of food intake by leptin. Here we show endogenous NTS LepR signaling is required for food intake and body weight control through a mechanism involving NTS LepR interactions with GI satiation signaling and AMPK activity.

AAV-mediated knockdown of LepR restricted to the mNTS and adjacent AP increased body weight gain compared to controls. The increased body weight gain was characterized by elevated fat mass and increased daily caloric intake, likely stemming from reduced sensitivity to vagally mediated satiation signaling and increased basal AMPK activity in the NTS. The chronic hyperphagia by LepRKD rats was observed when rats were maintained on either chow or HF diet, although the magnitude of the caloric hyperphagia (and increase in body weight gain) was greater with HF diet maintenance. The chronic hyperphagia was not associated with a compensatory increase in either core temperature or physical activity.

An interaction between CNS LepR signaling and the neural processing of GI satiation signals contributes to the intake inhibitory effects of leptin (Huo et al., 2007; Matson et al., 2000; Schwartz and Moran, 2002; Williams et al., 2006). The mNTS neurons are a primary site of this interaction. Current findings provide critical support for this mNTS-based hypothesis, showing that reduced mNTS/AP LepR signaling: (1) dramatically decreased sensitivity to the intake reducing effects of i.p. CCK administration (for both chow and 15% sucrose) and (2) increased the size of the first sucrose meal (10 min postsucrose presentation). The chronic hyperphagia by LepRKD rats was not limited to palatable food (i.e., sucrose and HF diet) but extended to chow diet. Other work from our laboratory shows that mNTS LepR signaling also interacts with the processing of gastric distension signals to control food intake (Huo et al., 2007). Thus, it is likely that the hyperphagia following mNTS/AP LepRKD results from a reduction in the potentiated signaling within mNTS neurons between leptin and various GI satiation signals.

The finding that pAMPKα2 levels were elevated in mNTS/AP micropunches of the LepRKD compared to shCtrl rats offers a

putative mechanism for the chronic hyperphagia of LepRKD rats and provides further support for the hypothesis that CNS AMPK signaling plays a key role in mediating leptin's effects on energy balance (Hayes et al., 2009; Minokoshi et al., 2004). Recently, hindbrain leptin administration was shown to reduce (1) AMPK activity in NTS-enriched tissue; and (2) food intake, in part by the suppression of AMPK activity (Hayes et al., 2009). Current results show that mNTS/AP LepRKD increased basal pAMPKα2 levels (correlated with an elevation in AMPK activity [Hayes et al., 2009; Minokoshi et al., 2004]) in mNTS/AP micropunches and suggest that the increased basal phosphorylation of AMPK is a consequence of chronically reduced LepR signaling. Elevated AMPK activity may provide a mechanism for the hyperphagia (Minokoshi et al., 2004) and for the decreased sensitivity to satiation signals observed in the LepRKD rats. Indeed, inhibition of hindbrain AMPK activity by fourth i.c.v. compound C reduced daily caloric intake of LepRKD rats to a level comparable to that of shCtrl rats. This dose of compound C had no intake effect in shCtrl rats with lower basal AMPK activity in the mNTS/AP, suggesting that LepRKD rats with higher basal AMPK activity are more sensitive to local AMPK inhibition.

Together with the lack of effect on core temperature and physical activity for mNTS/AP LepRKD rats, the hyperthermia and tachycardia effects observed following *exogenous* fourth i.c.v. (Skibicka and Grill, 2009) or mNTS parenchyma (our unpublished data) leptin injections suggest that NTS LepR signaling is sufficient in acute stimulation conditions but may not be necessary for the activity or thermic effects of endogenous LepR signaling. This notion is consistent with the perspective that CNS leptin-mediated control of energy expenditure is mediated by anatomically distributed networks and involves functional redundancy across other CNS LepR-expressing neurons.

Total adiposity of mNTS/AP LepRKD rats was greater than that of shCtrl rats, with the inguinal WAT mass accounting for the most significant change. Decreased NTS LepR-mediated sympathetic nervous system (SNS) tone to the inguinal WAT (Bamshad et al., 1998; Bowers et al., 2004) may explain the increase observed following mNTS/AP LepR knockdown, possibly through altered fat cell proliferation and/or decreased lipolysis within this depot. This hypothesis is supported by findings (Bowers et al., 2004; Foster and Bartness, 2006) showing that denervation of inguinal WAT results in a significant increase in fat cell mass and number. It also is possible that the increase in inguinal WAT seen in LepRKD rats is secondary to their chronic hyperphagia as a result of differential regulation of lipogenesis by CNS leptin signaling (Buettner et al., 2008), which also involves SNS mediation.

The qPCR results, histological confirmation of localized EGFP-expression in mNTS and AP neurons, and absence of LepR-expressing nuclei within the immediate vicinity of viral injections within the caudal brain stem (Huo et al., 2007; Li et al., 1999) indicate that the phenotype described results from reduction in LepR signaling by LepRKD only in the mNTS and AP. The recombinant AAV2 capsids used only transduce in local neuronal cell bodies and are not transported in a retro- or anterograde fashion (Burger et al., 2004). Thus, it is fair to assume that knockdown occurred only in LepR-expressing neurons of the mNTS and AP and that no alteration of LepR expression on astrocytes or glial cells of the NTS and AP (Dallaporta et al., 2009) occurred. Likewise, there was no alteration in any other CNS or vagal LepR-expressing nuclei that project to the NTS (Blevins et al., 2004; Peters et al., 2005; Zheng et al., 2005).

Rats with LepRKD in the VTA (Hommel et al., 2006), and mice with LepRKD restricted to VMN neurons (Dhillon et al., 2006), are hyperphagic under certain conditions, supporting a role for endogenous LepR signaling in food intake control in additional brain regions. Thus far, however, there is no indication that an alteration in satiation signal processing contributes to the hyperphagia of these models. In contrast, a case for a connection between ARC LepR signaling and satiation signal processing has been made (Morton et al., 2005). Those experiments show that the hyperphagia that drives the obesity of Koletsky rats is characterized by (1) an increase in meal size but not in meal frequency and (2) reduced sensitivity to the intake inhibitory effects of CCK. Together with current findings, the intake-inhibitory effects of endogenous CNS LepR signaling involve potentiation of the intake-inhibitory effects of GI satiation signals in at least two anatomically distributed nuclei, the ARC and mNTS.

Selective knockdown of LepR in mNTS and AP neurons significantly increased body weight gain and total adiposity and was attributed to chronic hyperphagia of maintenance chow and HF diet. This chronic hyperphagia was correlated with reduced sensitivity to the intake-suppressive effects of CCK and a basal increase in mNTS/AP AMPK activity. These data support the perspective of an anatomically distributed control of energy balance, whereby leptin signaling and AMPK activity in multiple CNS nuclei contribute to the overall control of food intake, energy expenditure, and body weight regulation. These results have implications for the development of pharmacological strategies in obesity research that would take advantage of the putative synergistic relationship between LepR and GI satiation signaling.

EXPERIMENTAL PROCEDURES

Subjects and Materials

Adult male Sprague Dawley rats (300–350 g; Charles River) individually housed in a room maintained at 23°C with 12 hr:12 hr light/dark cycle (08:00 hr lights on) had ad libitum access to chow (Purina Rodent Chow 5001) and water except as noted. All procedures conformed to the institutional standards of Animal Care and Use Committee (University of Pennsylvania).

The selective AMPK antagonist, compound C (Fisher Scientific), was chosen to examine hindbrain AMPK-mediated effects on food intake (Hayes et al., 2009; Kim et al., 2004). For the experiments addressing the interaction between NTS LepR signaling and GI-derived satiation signals, CCK (CCK-8; American Peptide, Inc.) was dissolved in sterile 0.9% saline and was administered via i.p. injections (1.0 ml/kg).

Design and Construction of shRNA and Viral Production, Purification, and Delivery

Hairpin RNA was designed to target specific regions of *Lepr* mRNA as described (Hommel et al., 2006). Viral production was accomplished using a triple-transfection, helper-free method and purified as described (Hommel et al., 2003, 2006). The virus was purified via iodixanol gradients as described (Hommel et al., 2006) and titered by infection of camptothecin-treated HT1080 cells.

Following 2 weeks of baseline behavioral and physiological testing, rats were very lightly anesthetized via ketamine (9.0 mg/kg), xylazine (0.27 mg/kg), and acepromazine (0.064 mg/kg). For bilateral mNTS/AP targeting, a total of 0.5 μl of purified virus (1–2 $\times$ 10^{11} infectious particles/ml) was delivered over a 5 min period per hemisphere via a Hamilton syringe connected to an injector

(Plastics One) that extended 2.0 mm below the tip of the caudal bilateral mNTS guide cannula (Supplemental Experimental Procedures; 7.9 mm ventral to the skull surface).

Tissue Collection

Ad libitum-fed rats were sacrificed by decapitation 1 hr into the light cycle. Inguinal, retroperitoneal, epididymal, and perirenal WAT depots were removed and weighed. Brains were rapidly removed, flash frozen in isopentane, and stored at −80°C until processing. Briefly, serial coronal sections (30 μm) of the caudal brain stem were mounted on a slide and viewed immediately under a fluorescent microscope (Nikon 80i) until EGFP-expressing neurons were visualized. Next, under 4× magnification, micropunches of the AAV-shLepR/shCtrl-infected mNTS/AP tissue were taken from two consecutive 80 μm frozen coronal sections. Tissue collected from one section was kept frozen for qPCR analysis of LepR and GAPDH, while tissue from the subsequent section was kept frozen for immunoblot analysis of pAMPKα2 and total AMPK. The next coronal section (30 μm) was again mounted on a slide to confirm EGFP presence. This process continued until micropunches were harvested for the entire mNTS/AP (at the AP level). Additionally, visualization of infected neurons continued until the presence of EGFP-expressing neurons was absent. Only data from rats with confirmed EGFP-expressing neurons in the mNTS/AP were used in final statistical analysis.

Immunoblot Analysis

mNTS/AP tissue lysates from ad libitum-fed, chow-maintained rats (7 weeks post-mNTS viral delivery) were prepared as described (Hayes et al., 2009; Minokoshi et al., 2004). Briefly, tissue lysates of LepRKD (n = 5) and shCtrl (n = 6) rats were transferred to PVDF membranes for immunoblot analysis. pAMPKα-Thr 172 rabbit monoclonal antibody (pAMPKα2; Cell Signaling; catalog number 2535S) was used to evaluate AMPK activity normalized to immunoblot analysis of total AMPKα (reactive against α1 and α2 subunits; Cell Signaling). Blots were quantified using NIH Image software.

RNA Isolation and Real-Time PCR

Total RNA was extracted from mNTS/AP tissue from ad libitum-fed, chow-maintained rats (7 weeks post-mNTS viral delivery) using Trizol (Invitrogen), and for further purification, the RNeasy kit (QIAGEN) was used. cDNA was synthesized from 1 μg total RNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). qPCR was done in duplicate using TaqMan Universal qPCR Master Mix (Applied Biosystems), and samples were run using the Eppendorf Mastercycler ep realplex. The primer/probe sets for LepR and GAPDH (internal control) were from Applied Biosystems (LEPR-Rn00664624_m1; GAPD-435293 2E, respectively). Relative mRNA expression was calculated using the comparative Ct method as described (Bence et al., 2006).

Feeding Behavior

For 2 weeks prior to AAV delivery, daily body weight and food intake measurements (±0.1 g) of rodent chow were made at 09:00 hr. In addition, rats (n = 9 shCtrl; n = 10 LepRKD) were trained to consume 15% (w/v) sucrose ad libitum for 1 hr (09:30–10:30 hr) for 1 week prior to baseline testing. Briefly, water and food were removed 20 min prior to testing. Five minutes prior to presentation of a calibrated tube containing 15% sucrose solution, rats received counterbalanced i.p. injections of CCK (3 μg/kg) or saline. Intakes were recorded (±0.1 ml) at baseline and every 10 min for 60 min, after which chow and water were returned. All injections were separated by a period of at least 48 hr.

Five days post-AAV delivery, behavioral testing of 15% sucrose intake following i.p. CCK or saline continued for the duration of the experiment. Five-and-a-half weeks post-AAV delivery, rats' (n = 9 shCtrl; n = 10 LepRKD) maintenance diet was switched to HF diet (Research Diets, D12492; 60% Kcal from fat), and daily measurements continued for an additional 3 weeks.

In a separate group of rats (n = 6 shCtrl; n = 6 LepRKD) implanted with fourth i.c.v. guide cannula (Supplemental Experimental Procedures), maintained exclusively on chow, and housed in a reverse-light cycle (lights on, 22:00 hr), additional food intake testing was conducted. Six weeks post-AAV delivery, intake measurements of chow following counterbalanced i.p. CCK (3 μg/kg) or saline delivery occurred as follows: 30 min predark cycle onset, food was removed. Animals received i.p. injections at dark cycle onset, preweighed chow was returned 5 min later, and intakes were measured 30 min postfood presentation. Data were analyzed as within-subject percent suppression of 30 min chow intake following CCK compared to intake following vehicle. At 3 weeks post-AAV delivery, rats underwent intake testing following counterbalanced fourth i.c.v. delivery of the AMPK inhibitor compound C (15 μg; dose subthreshold for effect in shCtrl rats from pilot studies) or DMSO (3 μl) (Hayes et al., 2009). Briefly, 30 min predark cycle onset, food was removed. Rats received fourth i.c.v. injections at dark cycle onset, and food was returned 15 min later. Feeding measurements occurred at 1, 3, 6, and 24 hr postfood presentation. For both CCK and compound C testing, all injections were separated by a minimum of 48 hr.

Data and Statistical Analyses

Data for each respective study were analyzed separately and expressed as mean ± SEM. For all experiments, comparisons between treatment means were analyzed by one- or two-way ANOVAs and, if appropriate, post hoc, pairwise comparisons were made using Tukey's honestly significant difference test with $p < 0.05$ considered statistically significant. Analyses were made using PC-SAS (version 8.02, SAS Institute) mixed procedure.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, Supplemental Results, two figures, and Supplemental References and can be found with this article online at doi:10.1016/j.cmet.2009.10.009.

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