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Leptin Regulates Energy Balance and Motivation Through Action at Distinct Neural Circuits

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Abstract

BACKGROUND—Overconsumption of calorically dense foods contributes substantially to the current obesity epidemic. The adiposity hormone leptin has been identified as a potential modulator of reward-induced feeding. The current study asked whether leptin signaling within the lateral hypothalamus (LH) and midbrain is involved in effort-based responding for food rewards and/or the modulation of mesolimbic dopamine.

METHODS—The contribution of endogenous leptin signaling for food motivation and mesolimbic dopamine tone was examined after viral-mediated reduction of the leptin receptor within LH and midbrain neurons in male rats.

RESULTS—Knockdown of leptin receptors selectively in the LH caused increased body weight, caloric consumption and body fat in rats maintained on a calorically dense diet. Knockdown of leptin receptors selectively in midbrain augmented progressive ratio responding for sucrose and restored high-fat diet-induced suppression of dopamine content in the nucleus accumbens.

DISCUSSION—In summary, endogenous leptin signaling in the hypothalamus restrains the overconsumption of calorically dense foods and the consequent increase in body mass, whereas leptin action in the midbrain regulates effort-based responding for food rewards and mesolimbic dopamine tone. These data highlight the ability of leptin to regulate overconsumption of palatable foods and food motivation through pathways which mediate energy homeostasis and reward respectively.

Keywords

Leptin; Lateral Hypothalamus; Midbrain; Mesolimbic Dopamine; Operant Responding

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The brain circuits regulating reward mechanisms and metabolism overlap, and compounds signaling adiposity level act upon both to modulate energy balance and reward behavior. For example, besides having a role in maintaining energy homeostasis, the adipose hormone leptin regulates reward-related behaviors, thus highlighting the potential for adiposity signals to modulate energy homeostasis and hedonics. Leptin administration increases the threshold for lateral hypothalamic self-stimulation (LHSS) (1) and reduces reinstatement of heroin self-administration (2). In addition to reducing food intake and body weight, exogenous leptin also decreases the acquisition and expression of high-fat diet-induced conditioned place preference (3) and motivated responding for palatable foods (4). Thus, leptin negatively modulates a spectrum of reward-related behaviors.

Leptin receptors are present in brain regions that modulate cognitive-emotional aspects of feeding behavior (5–8) including the lateral hypothalamus (LH), midbrain, and hippocampus. Of relevance, LH neurons which project to midbrain reward areas express leptin receptors (9), as do midbrain dopaminergic neurons (6), and when applied directly into the LH, leptin increases nucleus accumbens (NAcc) dopamine flux (9). However, administration of leptin into the midbrain inhibits the frequency of action potentials in dopamine neurons (10). Thus, leptin signaling at specific sites within the LH-midbrain circuitry may result in opposing actions on mesolimbic dopamine flux. The point is that while it is clear that leptin has the capacity to alter mesolimbic dopamine, it is unclear if endogenous leptin signaling within these regions is capable of modulating effort-based behaviors. To test this possibility, we utilized a genetic manipulation to reduce leptin signaling within the LH or midbrain of rats, and subsequently assessed operant responding and mesolimbic dopamine concentration.

METHODS AND MATERIALS

Animals

Male Long-Evans rats ($n = 5\text{--}8/\text{group}$) (Harlan, IN) weighing 250–300 g were housed individually in a vivarium with a 12:12 light/dark schedule and maintained at 25° C. All animals had *ad libitum* access to food and water throughout the study.

Diets

Rats receiving bilateral leptin injections were maintained on chow (Teklad, 3.41 kcal/g, 0.51 kcal/g from fat). Animals receiving viral injections had *ad libitum* access to one of two diets: chow (Chow) or high-fat diet (HFD) (Research Diets, New Brunswick, NJ, 4.41 kcal/g, 1.71 kcal/g from fat).

Apparatus

Operant procedures were conducted in identical conditioning chambers constructed of aluminum walls and Plexiglas sides measuring $21.6 \times 21.6 \times 27.9$ cm. A grid of 0.48-cm diameter stainless steel bars, spaced 1.9 cm apart, served as the floor of each chamber. A food cup was located on one wall of each chamber inside a 5×5 -cm recessed opening. Two levers were located approximately 3 cm to the left and right of the food cup. Only the right lever was active during this experiment. All experimental events were controlled and recorded by computers running ABET software (Lafayette Instruments; Lafayette, IN).

Operant Responding

In both the LH and midbrain studies, four groups of rats ($n = 8/\text{group}$) were injected with scrambled control virus or LepR virus (LepR) and maintained on either Chow or HF diet for 4 weeks prior to the beginning of the operant training. All training was conducted in the dark

and the animals were not food restricted during any phase of operant training. The reinforcer was a single 45-mg sucrose pellet (TestDiet, Richmond, IN). Operant training was carried out over 8 consecutive days with one 1-h trial per day. During the first two days of training, a fixed ratio (FR) 1 autoshaping procedure was employed, in which each lever press earned a single reinforcer. In addition, whenever 600 sec elapsed with no reinforcer delivery, a “free” sucrose pellet was dispensed into the food cup. All animals were then trained for 2 days using an FR1 schedule with no autoshaping component, followed by 2 days of FR3 training. At the conclusion of the 6-day operant training regimen, animals were given a single trial to lever press for sucrose under an incremental progressive ratio schedule of reinforcement where the lever press requirement for each subsequent reinforcer increased incrementally. The response requirements of the PR schedule increased through the following series: 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 178, 219, 268, 328, 402, 492, 693, 737, 901. The breakpoint for each animal was defined as the final completed requirement that preceded a 20-min period without responding.

Design and Construction of shRNA

We used shRNA sequences identical to those described by (Hommel et al., 2006). Lentivirus vector construction and packaging were performed by America Pharma Source, LLC (Rockville, MD). Briefly, scrambled (SCR) shRNA (Top, 5'-GATCCAGCACCATTTCGCTTCAATATTCAAGAGATATTGAAGCGGAAATGGTGCTTTTGG-3'-Bottom, 5'-AATTCCAAAAAGCACCATTTCGCTTCAATATCTTGAATATTGAAGCGGAAA TGGTGCTG-3') or shRNA directed against the rat leptin receptor (LepR) (Top, 5'-GATCCAGAAATCTTTAAATTACCATCATCTTCGAGATGATGGTAATTTAAAGATTTATTTTGG -3' Bottom, 5'-AATTCCAAAAATAAATCTTTAAATTACCATCATCTCGAAAGATGATGGTAATTTAAAGATTTCTG-3') were used. Both strands of oligonucleotides including linker and restriction sites were synthesized by Invitrogen. After annealing, the double strand shRNA DNA insert was ligated into lentivector (pHR-U6-EF-GFP) at BamHI and EcoRI sites directly, followed by DNA sequencing confirmation. GFP was used as selection marker driven by EF-1 α promoter. Lentiviruses were packaged by transfecting 293T cells, with vsv-g as envelope protein. The titers of the packaged virus for further experiments were in the range of $2-5 \times 10^9$ IU/mL. Cells were infected with the prepared lentivirus according to the protocol provided by America Pharma Source. Following in-vitro confirmation of gene knockdown, the virus was concentrated to approximately $1-2 \times 10^9$ infectious particles/ml.

qPCR

Rats were sacrificed via CO₂ asphyxiation, and brains were rapidly removed, frozen and stored at -80°C until processing. The LH and midbrain from each animal were microdissected using a AHP-1200CPV freezing plane (Thermoelectric Cooling America, Chicago, IL) which maintained a constant temperature of 12°C throughout the dissection process. High-quality mRNA was isolated by Trizol (Invitrogen, Carlsbad, CA) and chloroform (Sigma, St. Louis, MO) extraction. Complementary DNA was synthesized from 300 ng of mRNA by oligo DT priming (Invitrogen, Carlsbad, CA). cDNA were amplified in triplicate using 8 pg of each specific primer with quantification of the product by SYBR green fluorescence (Applied Biosystems, Foster City, CA). Leptin receptor was normalized to L32 control gene expression. All dissected tissue was further normalized using the viral reporter gene EGFP. EGFP mRNA expression was used as an internal positive control for both LH and midbrain infected tissue. Animals that did not express EGFP in the LH or midbrain were excluded from any further analysis.

Primers

The following primer sequences (IDT, San Diego, CA) were used to probe for rat leptin receptor, L32 and GFP, respectively: Leptin: 5' - AAT TGG AGC AGT CCA GCC TA - 3', 5' - TTT CCC ACA TCT TGT GAC CA - 3' and L32: 5' - CAG ACG CAC CAT CGA AGT TA - 3', 5' - AGC CAC AAA GGA CGT GTT TC - 3', GFP: 5' -GAC GTA AAC GGC CAC AAG TT- 3', 5' -AAG TCG TGC TGC TTC ATG TG- 3'.

Immunohistochemistry

Following viral delivery into the LH or midbrain animals were injected with an overdose of pentobarbital and perfused transcardially with ice cold saline for 1 min followed by 4% paraformaldehyde in 1× PBS for 20 min. Cryprotected brains were frozen and sectioned at 35 mm intervals. A full series of sections was double-labeled for pStat3 and GFP immunohistochemistry. The sections were incubated in the following solutions with the appropriate washes between solutions: 1% sodium hydroxide + 1% hydrogen peroxide/PBS, 0.3% glycine/PBS; 0.03% sodium dodecyl sulfate/PBS; Sections were then blocked by incubation in 4% horse serum with 0.4% Triton X-100. Rabbit anti-pStat3 (Cell Signalling Cat # 9145S) antibody diluted 1:100 in 4% horse serum with 0.4% Triton X-100 was applied to the sections for overnight incubation. The secondary antibody, biotinylated goat anti-rabbit IgG (1:250; Vector Laboratories; Burlingame, CA), was applied followed by incubation in avidin-biotin complex (1:500; Vectastain ABC; Vector Laboratories; Burlingame, CA) and an incubation in biotinyl tyramide signal amplification solution (1:250; PerkinElmer; Boston, MA). Cyanine 3 Streptavidin (Cy3; 1:200; Jackson Immuno Research; West Grove, PA) was then applied to the sections. The sections were then washed with PBS and incubated in 4% goat serum + 0.4% Triton X-100 blocking solution followed by rabbit anti GFP (Invitrogen Cat# A11122), formulated in the same blocking solution, overnight at RT followed by incubation in Alexa-488 goat anti-mouse IgG diluted (1:200; Invitrogen, Carlsbad CA, catalog # A11001). Slides were cover slipped with gelvatol mounting media containing DABCO antifade agent.

Dopamine Neurochemistry

Bilateral micropunches from the nucleus accumbens (NAcc) were dissected from each animal (Scrambled-Chow, Scrambled-HFD, LepR-Chow, LepR-HFD) under basal conditions. For high-performance liquid chromatography (HPLC) analysis, an antioxidant solution (0.4 N perchlorate, 1.343 mM ethylenediaminetetraacetic acid (EDTA) and 0.526 mM sodium metabisulfite) was added to the samples followed by homogenization using an ultrasonic tissue homogenizer (Biologics, Gainesville, VA). A small portion of the tissue homogenate was dissolved in 2% sodium dodecyl sulfate (SDS) (w/v) for protein determination (Pierce BCA Protein Reagent Kit, Rockford, IL). The remaining suspension was spun at 14,000g for 20 min in a refrigerated centrifuge. The supernatant was reserved for HPLC. Samples were separated on a Microsorb MV C-18 column (5 Am, 4.6×250 mm, Varian, Walnut Creek, CA) and simultaneously examined for DA, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), the latter two being markers of dopamine degradation, and for 5-HT and 5-HIAA to assess serotonin activity. Compounds were detected using a 12-channel coulometric array detector (CoulArray 5200, ESA, Chelmsford, MA) attached to a Waters 2695 Solvent Delivery System (Waters, Milford, MA) under the following conditions: flow rate of 1 ml/min; detection potentials of 50, 175, 350, 400 and 525 mV, and scrubbing potential of 650 mV. The mobile phase consisted of a 10% methanol solution in distilled H₂O containing 21 g/l (0.1 M) citric acid, 10.65g/l (0.075 M) Na₂HPO₄, 176 mg/l (0.8 M) heptanesulfonic acid and 36 mg/l (0.097 mM) EDTA at a pH of 4.1. Experimental samples were quantified against a 6-point standard curve with a minimum R² of 0.97. Quality control samples were interspersed with each run to ensure HPLC calibration.

Midbrain Cannulation Surgery

Rats ($n = 5/\text{group}$) were allowed to acclimate for 1–2 weeks before surgery. Under deep anesthesia, guide cannulae (Plastics One, Roanoke, Virginia) were implanted bilaterally in the midbrain. Coordinates for placement of the guide cannula in the midbrain were based on (Hommel et al., 2006) as follows: 5.7 mm posterior to bregma, 0.75 mm lateral from the midline and 7.8 mm ventral from the surface of the skull with lambda and bregma at the same vertical coordinate. Holes were drilled into the skull and self-tapping stainless steel screws were inserted. After placement, the 26-gauge guides were attached to the skull using cranioplastic cement (Plastics One). Once the surgery was complete sterile obturators (33gauge; 0.8 mm longer than the guide cannula) were inserted into the guides to reduce the potential for brain infection. For LH viral delivery, Hamilton syringe needles were targeted to 2.3 mm posterior from bregma, 2.0 mm lateral from the midline and 8.2 mm ventral to the skull surface. For midbrain viral delivery, syringe needles were targeted to 5.6 mm posterior from bregma, 2.2 mm lateral from the midline and 8.6 mm ventral from the surface of the skull. A total of 2.0 (LH) or 1.0 μl (midbrain) of purified virus ($1\text{--}2 \times 10^9$ infectious particles/ml) was delivered per side over a 5-min period.

Leptin Microinjection Procedure

After surgery, the rats were allowed to recover for two weeks before operant training began. Once PR was stable, injectors were inserted into the guides and the rats were placed into an open cage and allowed to move freely during the infusion. The infusion pumps were operated for 4 min, delivering 125 ng of leptin in 0.0125 μl or an equivalent volume of vehicle per min, per side. Leptin (R&D Systems, Torrance, CA) was dissolved according to manufacturer's protocol. The injectors were left in the brain for an additional minute after the end of the injection before being removed and replaced with obturators.

Determination of Cannula Placement

At the completion of the infusion experiments, animals were injected with leptin (500 ng/side) directly into the VTA and sacrificed 45 min later via transcardial infusion of 4% paraformaldehyde. Brain sections were then analyzed by histology for phospho-STAT3 (p-STAT3) staining to evaluate the exact placement relative to tyrosine hydroxylase (TH) staining, the rate limiting enzyme regulating dopamine production. Brains from rats injected with leptin that did not display p-STAT3 staining within the midbrain region: bregma-5.0 through bregma-6.0 were not included in further analyses; this resulted in the rejection of two animals from the behavioral studies.

Statistical analysis

Data were analyzed using STATISTICA version 6.0 for PCs. Operant responding, leptin receptor expression and mesolimbic dopamine levels were analyzed using analysis of variance (ANOVA). A least significant differences (LSD) post-hoc comparison was used to assess the source of significant main effects and interactions.

RESULTS

Histological confirmation of Scrambled shRNA and LepR shRNA -injected rats identified infection of LH and midbrain neurons by expression of enhanced green fluorescent protein (EGFP expressed by the lentiviral vector) (Figures 1A & B). Double-labeled immunofluorescence staining of LH-injected rats revealed a very low number of orexin neurons infected after viral injection (Figure 1D). In the LH, the viral spread around the injections site extended 0.4mm in both the rostral and caudal direction infecting the LH from bregma -1.9 to bregma -2.7 . The midbrain injections targeted the ventral tegmental area

(VTA) as well as the medial and lateral aspects of the substantia nigra (SN), both of which express the long form of the leptin receptor⁸. In the midbrain, infected neurons were positive for tyrosine hydroxylase (TH), indicating that viral delivery within the midbrain infected mesolimbic dopamine neurons (Figure 1E). Viral spread in the midbrain spanned from bregma -5.5 to bregma -5.9 relative to the injection site. EGFP-positive micropunches were analyzed using quantitative real time PCR (qPCR) to confirm knockdown of leptin receptors (see Supplement: Figures S2 and S3). Representative qPCR analysis of EGFP-infected LH (n = 8) and midbrain (n = 7) tissue revealed a 30% decrease in LepR mRNA in neurons infected with the LepR^{shRNA} construct in the LH and a 35% decrease in the midbrain (Figure 1C). In addition, reduction of LepR in both the LH and midbrain resulted in decreased pSTAT-3 signaling in infected regions suggesting that this manipulation resulted in functional loss of leptin receptor signaling (see Supplement: Figure S1).

Rats with reduced LepR in the LH (n = 8) displayed a significant increase in caloric consumption when maintained on HFD, an effect that became apparent within the second and third weeks after viral delivery (Figure 2B). Interestingly, rats with reduced midbrain LepR (n = 8) had an acute increase in HFD consumption which was present only during the first four days of diet exposure (Figure 4B). There were no effects of LepR reduction on consumption of chow in either group (Figures 2 and 4B).

LepR reduction in the LH resulted in increased body weight gain in HFD rats, an effect which appeared 15 days after viral delivery and persisted throughout the remainder of the study (Figure 2A). At the conclusion of the experiment, NMR analysis revealed a significant increase of body fat in LH LepR^{shRNA}-treated rats (Figure 2C) compared with Scrambled^{shRNA} control rats. In contrast, there were no differences in body weight or body fat in rats with targeted midbrain LepR^{shRNA} injections (Figure 4A). These results suggest that leptin signaling within the LH is capable of counter-regulating the effects of calorically dense foods on body weight gain.

To determine if leptin signaling within LH-midbrain circuitry modulates effort-based feeding, operant responding was assessed. Rats with reduced LH LepR made a similar number of responses for sucrose when responding on a fixed (FR1, SCR 41.15 $\pm$ 15.08 vs LepR 32.14 $\pm$ 11.5, FR3, SCR 33.2 $\pm$ 12.6 vs LepR 32.8 $\pm$ 8.85) or progressive ratio (PR) schedule of reinforcement compared to Scrambled^{shRNA} control rats (Table 1, Figures 3A&B). In contrast, reduction of leptin receptors within the midbrain augmented the break point (Figure 5A). Reduction of leptin receptor in the midbrain had no effect on fixed ratio responding (FR1, SCR 43.6 $\pm$ 6.13 vs LepR 60.6 $\pm$ 10.7, FR3 SCR 118.8 $\pm$ 20.5 vs LepR 163.7 $\pm$ 34.7). Because midbrain LepR reduction augmented operant responding, we next assessed the effects of direct leptin infusion within the midbrain on PR responding. Bilateral injection of 500 ng of leptin directly into the midbrain activated the JAK-STAT signaling pathway and resulted in p-STAT3 activation. Activated dopaminergic neurons were assessed by STAT3 phosphorylation. A survey of midbrain injected tissue revealed that p-STAT3 positive neurons were present from -5.0 bregma to -6.0 bregma. p-STAT3 neurons were visualized in both the VTA and SN throughout this rostro-caudal extent within midbrain tissue. In addition, p-STAT3 positive neurons were present in midline structures including the linear raphe and parabrachial pigmented nucleus. Similar to previous reports (10) the majority of neurons expressing p-STAT3 were positive for tyrosine hydroxylase (TH) indicating that midbrain injection of leptin activated leptin receptors present on dopaminergic neurons (Figure 5F).

To determine if knockdown of LH or midbrain leptin receptors altered mesolimbic dopamine, we utilized a model in which mesolimbic dopamine is reduced after prolonged exposure to a HFD. HFD rats typically display decreased dopamine turnover within the

NAcc after prolonged diet exposure (11). In the current study, HFD rats with reduced LH LepR displayed similar NAcc dopamine levels relative to Scrambled ^{shRNA} rats (Figure 3C). In contrast, knockdown of leptin receptors within the midbrain rescued the effect of HFD on NAcc dopamine (Scrambled ^{shRNA} = 59 ± 8% of control while LepR ^{shRNA} = 90 ± 5% of control) supporting the contention that leptin signaling within midbrain neurons mediates the effects of HFD on mesolimbic dopamine concentration (Figure 5C).

DISCUSSION

The goal of the current study was to test the hypothesis that leptin signaling within hypothalamic/midbrain circuitry regulates effort-based feeding. In addition, we assessed the ability of leptin to modulate mesolimbic dopamine. Several significant findings emerged. Leptin signaling in the lateral hypothalamus regulates food intake, body weight gain and the deposition of body fat in animals fed a calorically-dense diet without affecting effort-based responding for food rewards or mesolimbic dopamine concentration. In contrast, leptin signaling in the midbrain augmented operant responding for sucrose and restored mesolimbic dopamine in animals maintained on a high-fat diet, an effect that is maximal with basal (physiological) leptin signaling. Collectively, these findings suggest that leptin acts within distinct neuronal circuits to manifest its effects on metabolism and motivation.

The ability of leptin to regulate energy balance has been studied extensively within the mediobasal hypothalamus (12–15). However leptin receptors are also present in the lateral hypothalamus, a region that serves as a nexus for feeding and food reward (16). LH leptin receptors are functionally active, as assessed by phospho-STAT-3 activation, and direct application of leptin into the LH decreases food intake and body weight in rodents (9), suggesting that LH leptin signaling has the potential to play a regulatory role in the mediation of energy homeostasis. The current data support this model. Reduction of leptin receptors in the LH led to increased food intake, body weight gain and body fat deposition, an effect that was present only when animals were maintained on a HFD. In contrast, previous studies reported that reduced leptin signaling in hindbrain nuclei yields increases in food intake, body weight and body fat in animals maintained on low-fat chow (17). Thus, while leptin is capable of regulating energy balance through action at both hindbrain and hypothalamus, the present data raise the possibility that LH leptin signaling regulates body weight homeostasis in a macronutrient-dependent fashion.

Reduction of leptin receptor expression within the midbrain did not affect body weight in animals maintained on low-fat chow, which agrees with prior studies (10). Importantly, in the present study, animals with reduced midbrain leptin receptor expression maintained on HFD did not display significant weight gain compared with controls. This aspect is particularly interesting given our observation that reduction of leptin receptor expression within this region increases acute intake of HFD, suggesting that leptin signaling in the midbrain decreases sensitivity to highly palatable foods. Thus, one might predict that maintenance on such a diet would lead to weight gain in excess of that normally observed in animals fed HFD *ad libitum*. However, over the four weeks of access to the HFD, animals with reduced midbrain leptin receptor gained similar weight as control animals.

In the context of food reinforcement, the present data suggest that leptin signaling within midbrain or LH neurons is capable of modulating differential aspects of food intake behavior. The data also imply that the maximal effects of leptin on operant responding occur with basal leptin. Thus, reducing midbrain leptin receptor expression increased progressive ratio responding rates, a task dependent upon intact dopamine function (20). However, activation of midbrain leptin receptors with exogenous leptin had no effect on progressive ratio (PR) responding. Direct application of exogenous leptin into the VTA does attenuate

activation of midbrain dopaminergic neurons (10), and dopamine release within the NAcc is correlated with both appetitive and consummatory aspects in food-reward paradigms (18,19). One interpretation of these collective results is that transient elevations in midbrain leptin are capable of modulating feeding behavior and NAcc dopamine flux; however, the ability of midbrain leptin to modulate effort-based responding for food requires more prolonged action such as that achieved through reduction of leptin receptor expression.

Because progressive ratio responding is modified by dopamine, suggesting that LepR reduction would alter mesolimbic dopamine levels, we hypothesized that a reduction of leptin receptor within both the LH and midbrain would prevent the effects of HFD on NAcc dopamine. Surprisingly, reduction of LepR within the lateral hypothalamus did not alter NAcc dopamine in any group tested. The lateral hypothalamus is capable of modulating NAcc dopamine levels through the synaptic modulation of dopaminergic neurons in the VTA by orexin (21). This possibility is consistent with the demonstration that central leptin increases the threshold for lateral hypothalamic self-stimulation in addition to attenuating operant responding and conditioned place preferences for food rewards (1,3,4). Within the LH, LepR-expressing neurons directly innervate the VTA (9), and central leptin administration decreases lateral hypothalamic orexin tone (22). Collectively, these data suggest that leptin acting within the LH is capable of modulating NAcc dopamine. Although reduction of LepR within the LH in the present study did not alter NAcc dopamine levels, it is possible that obtaining a real-time dopamine measurement may be necessary to monitor synaptically-mediated changes in NAcc dopamine. However, reduction of midbrain leptin receptor restored dopamine levels to control values in rats treated with HFD. These data are supported by the observation that midbrain leptin administration decreases the firing rate of dopaminergic neurons (10) and suggest that leptin signaling in the midbrain is capable of influencing mesolimbic dopamine neurochemistry in models of obesity.

The mesolimbic dopamine system is activated in part by the LH orexin system, and this idea has been validated in the context of both food reward and relapse to psychostimulants (21,23,24). It is intriguing that leptin acting within the LH decreases hypothalamic orexin tone, and when applied to the midbrain attenuates the firing of dopaminergic neurons, suggesting that in periods of positive energy balance, leptin may be capable of modulating brain reward circuits through action at both neural substrates. The present data indicate that leptin signaling within the LH regulates energy homeostasis and metabolism, whereas midbrain leptin modulates effort-based responding for food and mesolimbic dopamine. When viewed collectively, these data suggest that leptin acts at two distinct neural substrates to regulate metabolism and motivation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Leptin receptor knockdown in the LH and midbrain

(A and B) Low-power immunofluorescent analysis depicting the extent of lenti-viral infection, with identification of EGFP-expressing neurons in the (A) LH and (B) midbrain brain nuclei that express the LepR. EGFP-expressing neurons were present in the LH, lateral VTA, and substantia nigra (SNM and SN). Scale Bar: 200 μ m.

(C) Representative qPCR analysis revealed a significant reduction in LepR mRNA in LH/ Midbrain micropunched tissue for LepR shRNA-treated rats compared to Scrambled control rats. **= ($p < 0.0024575$ & $p < 0.0016894$). Error bars represent $\pm$ s.e.m. (D and E) High-power immunofluorescent analysis illustrating the extent of (D) orexin and (E) dopamine neuronal infection in LepR-treated rats. Both orexin (D) and Tyrosine Hxdroxylase (TH) (E) are immunostained red. Scale Bar: 200 μ m.

Abbreviations: AHP: Anterior Hypothalamic Area, Posterior Part, AHC: Anterior Hypothalamic Area, Central Part, F: Fornix, LH: Lateral Hypothalamic Area, PN:Paranigral Nucleus, VTA: Ventral Tegmental Area, SNM: Substantia Nigra Medial Part, SN: Substantia Nigra.

Figure 2. Reduction of *lepr* in the LH Increases Body Weight, Caloric Consumption and Body Fat

(A) Body weight change in LepR and Scrambled treated rats maintained on either chow or high fat diet (HFD). * = ($p < 0.0015379$) indicates significant differences between Scrambled and LepR-treated rats maintained on HFD.

(B) Caloric consumption in LepR and Scrambled treated rats maintained on either chow or HFD. * = ($p < 0.000117$) indicates significant differences between Scrambled and LepR-treated rats maintained on HFD.

(C) NMR body fat analysis in LepR and Scrambled treated rats after thirty days of exposure to chow or HFD. * = ($p < 0.000433$) relative to chow, ** = ($p < 0.0031673$) indicates significant differences between LepR and Scrambled-treated rats maintained on HFD.

Figure 3. Reduction of LepR in the LH does not affect PR responding or mesolimbic dopamine (A) Fixed and (B) Progressive Ratio (PR) break point responding in LepR and Scrambled treated rats maintained on chow.

(C) Basal dopamine levels in the nucleus accumbens (NAcc) from LepR and Scrambled treated rats maintained on chow or HFD. * $(p < 0.017784)$ indicates significant differences between Scrambled treated rats maintained on Chow or HFD. * $(p < 0.039399)$ indicates significant differences between LepR treated rats maintained on Chow or HFD. Error bars represent $\pm$ s.e.m.

Figure 4. Leptin receptor knockdown in midbrain neurons does not affect body weight or caloric consumption

(A) Body weight change in LepR and Scrambled treated rats maintained on either chow or high fat diet (HFD).

(B) Caloric consumption in LepR and Scrambled treated rats maintained on either chow or HFD. *=($p < 0.0009431$) indicates significant differences between Scrambled and LepR-treated rats maintained on HFD after four days of diet exposure.

Figure 5. Leptin receptor knockdown in midbrain neurons augments effort-based responding for food rewards

(A) Fixed and (B) Progressive Ratio (PR) break point responding in LepR and Scrambled treated rats maintained on chow. * = ($p < 0.0022121$).

(C) Basal dopamine levels in the nucleus accumbens (NAcc) from LepR and Scrambled treated rats maintained on chow or HFD. * = ($p < 0.003456$) indicates significant differences between Scrambled treated rats maintained on chow or HFD. ** = ($p < 0.009563$) indicates significant differences between Scrambled and LepR-treated rats maintained on HFD. Error bars represent $\pm$ s.e.m.

(D) PR break point responding in rats injected bilaterally with 500ng of leptin. Error bars represent $\pm$ s.e.m.

(E) Double-label fluorescent immunostaining of TH and pSTAT3 taken 1 h after a 500 ng dose of saline or leptin (F) administered directly into the midbrain. Scale Bar: 200 μ m. TH immunostaining is green, pSTAT3 staining is red.

Table 1

Detailed description of the number of earned reinforcers, total lever presses and average time spent in operant chambers from rats treated with scrambled control virus (SCR) or leptin receptor shRNA (LepR shRNA) when responding under a progressive ratio schedule of reinforcement.

Brain Region	Group	Earned Reinforcers	Total Responses	Avg Time in Chamber (min)
LH	SCR	6.28 $\pm$ 1.52	68 $\pm$ 17.53	105 $\pm$ 11.17
LH	<i>LepR shRNA</i>	7 $\pm$ 1.21	57 $\pm$ 10.16	98 $\pm$ 7.62
Midbrain	SCR	8 $\pm$ 0.44	71 $\pm$ 10.07	117 $\pm$ 9.05
Midbrain	<i>LepR shRNA</i>	11.5 $\pm$ 0.55 *	205 $\pm$ 16.05 *	128 $\pm$ 5.03

*
($p < 0.028174$).