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Leptin Receptor Signaling in Midbrain Dopamine Neurons Regulates Feeding

Jonathan D. Hommel,[2] Richard Trinko,[1,2] Robert M. Sears,[1] Dan Georgescu,[2] Zong-Wu Liu,[3,4] Xiao-Bing Gao,[4] Jeremy J. Thurmon,[2] Michela Marinelli,[5] and Ralph J. DiLeone[1,2,*]

[1] Departments of Psychiatry and Neurobiology
Yale University School of Medicine
New Haven, Connecticut 06519

[2] Department of Psychiatry
University of Texas Southwestern Medical Center
Dallas, Texas 75390

[3] Department of Neurobiology
Yunyang Medical College
Shiyan, Hubei, 442000
China

[4] Department of Obstetrics & Gynecology
Yale University School of Medicine
New Haven, Connecticut 06520

[5] Department of Cellular and Molecular Pharmacology
Rosalind Franklin University of Medicine and Science
North Chicago, Illinois 60064

Summary

The leptin hormone is critical for normal food intake and metabolism. While leptin receptor (*Lepr*) function has been well studied in the hypothalamus, the functional relevance of *Lepr* expression in the ventral tegmental area (VTA) has not been investigated. The VTA contains dopamine neurons that are important in modulating motivated behavior, addiction, and reward. Here, we show that VTA dopamine neurons express *Lepr* mRNA and respond to leptin with activation of an intracellular JAK-STAT pathway and a reduction in firing rate. Direct administration of leptin to the VTA caused decreased food intake while long-term RNAi-mediated knockdown of *Lepr* in the VTA led to increased food intake, locomotor activity, and sensitivity to highly palatable food. These data support a critical role for VTA *Lepr* in regulating feeding behavior and provide functional evidence for direct action of a peripheral metabolic signal on VTA dopamine neurons.

Introduction

An imbalance between the natural drive to feed and excess resources has contributed to the increased rates of obesity (Mokdad et al., 2003). In order to understand this problem and to design effective treatments, the molecular and neural mechanisms by which the brain regulates food intake need to be better defined. In the last ten years, great progress has been made in the identification of peripheral signals that communicate metabolic information to the brain. In particular, the identification of leptin, the gene mutated in the *obese* (*ob*) mouse, initiated a highly productive stage of feeding research.

Leptin is a secreted protein expressed in adipocytes and is a critical signal that conveys levels of peripheral fat stores to the brain (Frederich et al., 1995; Zhang et al., 1994). Further work, including the cloning of *Lepr* (Chen et al., 1996; Tartaglia et al., 1995), demonstrated that the ventral hypothalamus (arcuate nucleus) is an important mediator of the actions of leptin in the brain (Elmquist et al., 1998; Huang et al., 1996). An elegant model has been proposed whereby *Lepr* activity inhibits the orexigenic neurons while exciting the anorexigenic neurons within the arcuate nucleus (Cowley et al., 2001). Ongoing work is identifying the role that hypothalamic neuronal populations play in controlling metabolism and body weight in response to leptin (Balthasar et al., 2004; Dhillon et al., 2006).

While most studies have investigated leptin signaling in the hypothalamus, there is evidence that leptin has direct effects in other brain regions. Extrahypothalamic *Lepr* expression is seen in the hippocampus, brain stem, cortex, and midbrain (Elmquist et al., 1998). Moreover, leptin administration to the brain stem has been shown to modulate feeding behavior (Grill et al., 2002), while administration to the hippocampus enhances synaptic plasticity (Shanley et al., 2001). These results indicate that leptin function can be mediated via direct signaling to extrahypothalamic regions of the brain.

Expression of Lepr in the dopamine neurons of the midbrain (Figlewicz et al., 2003) suggests a potential molecular circuit that would connect peripheral metabolic signals directly to mesolimbic brain circuits that regulate motivational or hedonic elements of ingestive behavior (Figlewicz, 2003; Saper et al., 2002). The VTA dopamine neurons have been well studied for their role in drug addiction (Kauer, 2004; Marinelli et al., 2006; White and Kalivas, 1998), while pharmacological manipulations within the VTA have demonstrated effects on feeding behavior (Badiani et al., 1995; Mucha and Iversen, 1986; Noel and Wise, 1995). Intriguingly, existing data demonstrate that leptin is able to modulate reward-seeking behavior and drug relapse (Figlewicz et al., 2004; Fulton et al., 2000; Shalev et al., 2001), behaviors known to depend on the function of mesolimbic dopamine circuits. Likewise, changes in leptin levels could affect feeding behavior via direct action on VTA dopamine neurons. Here, we present data demonstrating that *Lepr*-mediated signaling occurs in dopamine neurons and that modulation of VTA *Lepr* signaling can modify dopamine neuron activity and food intake.

Results

Expression of *Lepr* mRNA in VTA Dopamine Neurons

Previous work using fluorescent immunohistochemistry suggested that the Lepr protein is expressed within dopamine neurons of the VTA (Figlewicz et al., 2003). We used double-label fluorescent in situ hybridization to identify the transcripts of both *Lepr* and tyrosine hydroxylase (TH) simultaneously. TH is an enzyme in the dopamine synthesis pathway and therefore serves as a marker of dopamine neurons. We observed staining of both *Lepr* mRNA and TH mRNA within the VTA (Figure 1A). Extensive colocalization of *Lepr* and TH is apparent under

*Correspondence: ralph.dileone@yale.edu

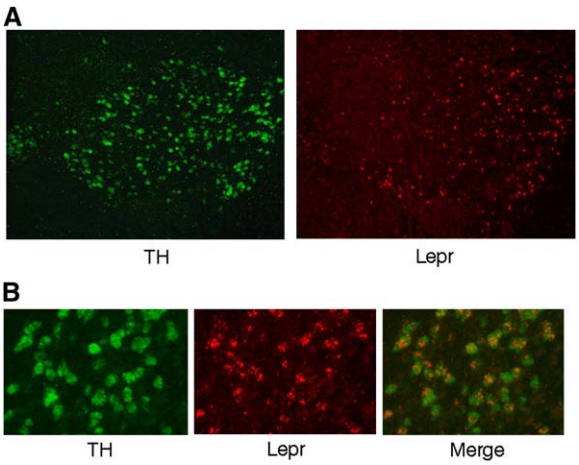

Figure 1. *Lepr* mRNA Is Expressed in Dopamine Neurons of the Rat VTA

(A) Low-power image of double label fluorescent in situ hybridization showing expression of TH mRNA (green) and *Lepr* mRNA (red) in the VTA.

(B) High-power image showing expression of TH and *Lepr* mRNA. Colocalization of the two mRNAs is seen in the merged panel.

high magnification (Figure 1B), suggesting that Lepr mRNA is expressed in dopamine neurons. While it remains possible that some nondopaminergic neurons (e.g., GABAergic interneurons) in the VTA express *Lepr*, >90% of neurons with *Lepr* mRNA are dopamine neurons (Figure 1B). In addition, ~60% of the dopamine neurons express detectable *Lepr* mRNA (Figure 1). These *Lepr* mRNA localization results, together with previous protein localization data (Figlewicz et al., 2003), suggest that leptin could have a major impact on overall VTA function.

Lepr Signaling in the VTA Increases Phosphorylation of STAT3

In order to assess whether VTA *Lepr* is functional, the JAK-STAT signaling pathway was evaluated with phosphoprotein analysis. This signaling cascade is activated upon leptin binding to *Lepr* in the hypothalamus (Ghilardi et al., 1996; Vaisse et al., 1996), resulting in the phosphorylation of STAT3. Mice with point mutations in *Lepr* that block the binding and phosphorylation of STAT3 become severely hyperphagic and obese, a phenotype that closely resembles mice with a *Lepr* null mutation (Bates et al., 2003).

To determine if leptin causes phosphorylation of STAT3 (pSTAT3) in the VTA, animals were injected with leptin (3 mg/kg intraperitoneal, within dose-response range for phosphorylation of hypothalamic STAT3, see Vaisse et al., [1996]). Western blot analysis on VTA tissue collected 2 hr after leptin administration revealed increased phosphorylation of pSTAT3 at tyrosine residue 705 (Figure 2A). Although this phosphorylation data suggest that circulating leptin can activate part of the JAK-STAT pathway in the VTA, we could not rule out indirect activation of JAK-STAT as a result of primary leptin action on the hypothalamus. To asses whether leptin can directly increase phosphorylation of VTA STAT3, leptin was delivered directly to the VTA of rats via surgically

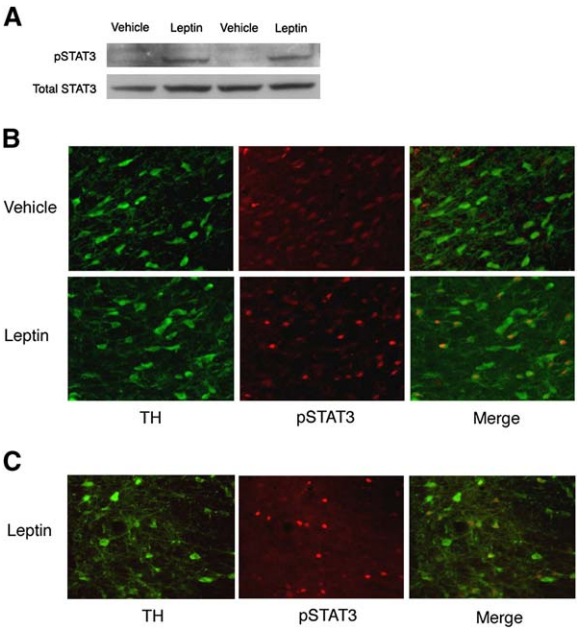

Figure 2. Leptin Induces JAK-STAT Signaling in Midbrain Dopamine Neurons

(A) Western blot analysis showing pSTAT3 (lower band in upper panel) and total STAT3 (lower panel) in the VTA of mice injected with either vehicle or leptin (3 mg/kg i.p.)

(B) Double-label fluorescent immunostaining of TH and pSTAT3 from tissue taken 45 min after infusion of vehicle or leptin (1 μg) to the VTA of awake, free-moving rats. TH immunoreactivity (green) is colocalized with pSTAT3 immunoreactivity (red, nuclear) only in animals that received leptin (lower right panel).

(C) Double-label fluorescent immunostaining of TH and pSTAT3 from tissue taken 1 hr after intracerebroventricular infusion of vehicle or leptin (5 μg) in awake, free-moving rats.

implanted bilateral cannulae. Robust pSTAT3 labeling was observed within the VTA 45 min after infusion of leptin (Figure 2B; see Figure S1 in the Supplemental Data available with this article online). Moreover, the pSTAT3 staining was found in TH-positive neurons, indicating that the dopamine neurons are the primary population of neurons responding to the administered leptin, in agreement with the localization of *Lepr* on TH-positive neurons.

These data indicate that leptin can directly activate the JAK-STAT pathway within VTA dopamine neurons. In order to more effectively quantify the cell populations responding to the leptin, we assessed pSTAT3/TH colocalization throughout the rostrocaudal extent of the VTA following intracerebroventricular (ICV) leptin infusions (Figure 2C). A survey indicated that most, but not all, of the VTA neurons responding were dopaminergic: at −5.2 bregma, 82% (73/89) were TH positive; at −5.6 bregma, 80% (80/100) were TH positive; at −6.0 bregma, 95% (95/100) were TH positive.

Leptin Infusion Directly into the VTA Suppresses Food Intake

To evaluate the role of *Lepr* signaling in the VTA, a series of doses of leptin (100 ng, 330 ng, or 1 μg, based on data from Grill et al., [2002] and Satoh et al., [1997]) was administered to the VTA, and food consumption was

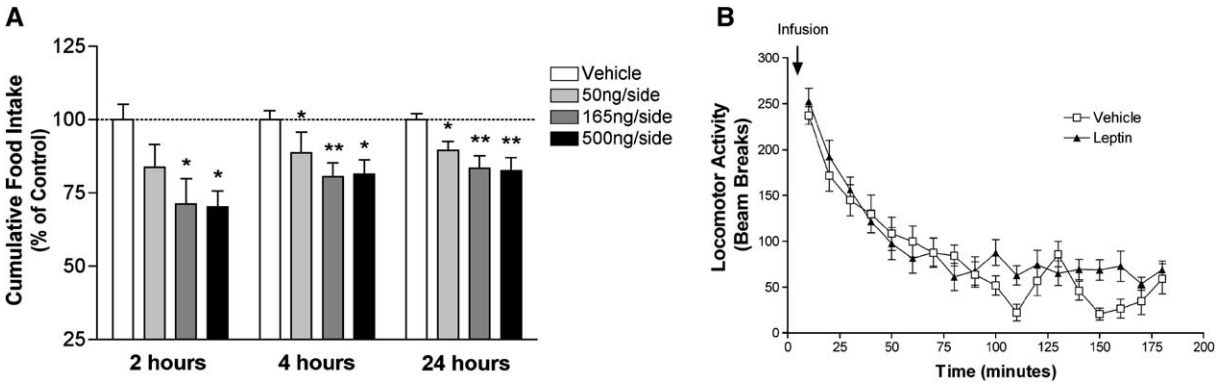

Figure 3. Direct Leptin Administration to the VTA Results in a Reduction in Food Intake

(A) Food intake expressed as a percentage of control over 24 hr following infusion of leptin directly into the VTA of awake, free-moving rats. Animals received infusions of vehicle (white bars, n = 36), 100 ng (light gray bars, n = 10), 330 ng (dark gray bars, n = 10), or 1000 ng of leptin (black bars, n = 16). Food intake measurements began 30 min after completion of infusions and total intake was measured at 2 hr, 4 hr, and 24 hr.

(B) No significant difference in locomotor activity was observed over 3 hr when comparing infusion of either vehicle (open squares, n = 6) or leptin (closed triangles, n = 6) at a dose of 500 ng/side. (*$p < 0.05$; **$p < 0.01$ by ANOVA followed by post hoc tests).

Error bars represent the standard error of the mean (SEM).

monitored over a 24 hr period beginning at the onset of the dark cycle. A summary of cannula placements used in this analysis is shown in Figure S2. All doses of leptin caused a significant decrease in food intake over the first 4 hr (treatment main effect, $F_{(3,71)} = 15.18$, $p < 0.001$; treatment x time interaction, $F_{(6,194)} = 2.3$, $p < 0.05$; Figure 3A), and the suppression of feeding was maintained over 24 hr of cumulative food intake. Infusion of 330 ng (165 ng/side) of leptin inhibited food intake by approximately 30% over the first 2 hr of feeding. Doses higher than 330 ng did not further inhibit feeding, suggesting a maximal leptin effect at this dose range. The observed decrease in food intake is consistent with the anorectic effects of systemic or ICV leptin (Halaas et al., 1995; Seeley et al., 1996).

The VTA also has a role in controlling locomotor and exploratory behavior (Badiani et al., 1995; Mogenson and Yang, 1991) raising the possibility that *Lepr* signaling to midbrain dopamine neurons could effect general activity. To address this question, ambulation was measured following delivery of leptin to the VTA. At the onset of the dark cycle, rats received either vehicle alone or 1 μg (500 ng/side) of leptin directly to the VTA, and their activity was assessed. No significant differences in the number of beam breaks were observed over a 3 hr period following the infusions (Figure 3B), suggesting that the changes in feeding were not the result of alterations in general locomotor activity.

Leptin Inhibits Firing of VTA Dopamine Neurons In Vivo

To test if circulating leptin could affect the electrophysiological properties of neurons in the VTA, the frequency of dopamine cell firing was observed following leptin administration. Extracellular, single-unit recordings were made in the VTA of anesthetized rats before and after intravenous delivery of 2 mg/kg leptin. Dopamine neurons were identified by use of characteristic electrophysiological properties (Henry et al., 1989; Marinelli et al., 2006; Marinelli and White, 2000; Ungless et al., 2004; see Experimental Procedures for details), and anatomical placement was confirmed by histological analysis (Figure S3). Leptin caused an ~40% decrease in the frequency of dopamine cell firing when compared to baseline or saline (treatment main effect, $F_{(1,66)} = 8.65$; $p < 0.001$; Figure 4). This effect appeared 10 min after leptin delivery and remained consistent up to 35 min (time main effect, $F_{(5,41)} = 5.1$, $p < 0.001$; treatment × time interaction, $F_{(5,66)} = 2.01$, $p = 0.08$; Figure 4). The decrease in firing rate was not due to reduced recording stability, as activity was stable over time for animals receiving saline (Figure 4, white bars; time main effect, $F_{(5,25)} = 0.5$, $p = 0.81$).

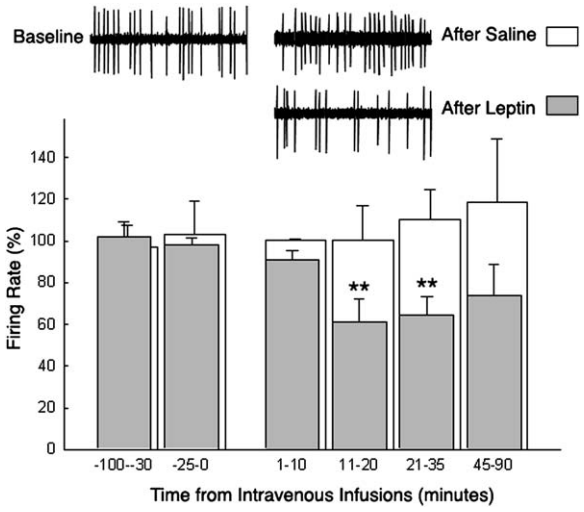

Figure 4. Peripheral Administration of Leptin Results in Reduced VTA Dopamine Neuronal Firing in Anesthetized Rats

The firing frequency decreased by 40% from 11 to 35 min after leptin injections. Representative 5 s single-unit traces are shown from neurons recorded after leptin or saline administration compared to baseline (top panels, see Experimental Procedures for details). The graph shows summary data for both saline-treated (white bars) and leptin-treated (gray bars) animals and represents 78 total neurons from ten rats. (**$p < 0.01$ by ANOVA followed by post hoc test). Error bars represent SEM.

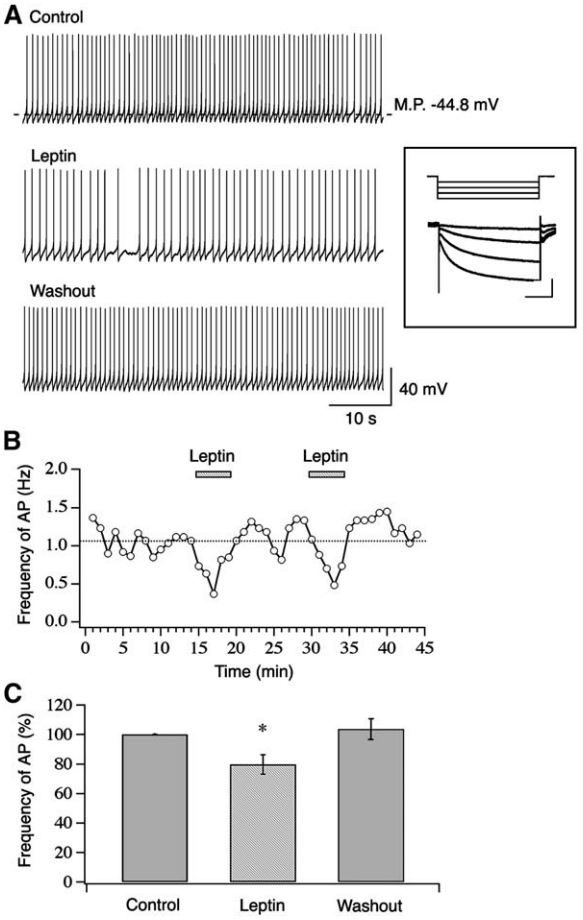

Figure 5. Leptin Decreases the Frequency of Action Potentials in Dopamine Neurons in the VTA

(A) Sample traces recorded before, during, and after application of leptin (100 nM) from a dopamine neuron under current clamp (M.P. = −44.8 mV). The inset demonstrates the *I*h current used to identify dopamine neurons; dopamine neurons exhibit the presence of a large *I*h current, which is distinctive from nondopamine neurons nearby. The *I*h currents (lower traces) were induced by a series of voltage steps from −60 mV to −100 mV (upper traces) in neurons under voltage clamp held at −50 mV. Inset scale bar: 200 pA, 0.5 s.
(B) A time course of the effect of leptin on the frequency of action potentials. The effect of leptin was fast and reversible; the bars above the trace indicate the multiple applications of leptin.
(C) Pooled data showing a reduction in action potentials (n = 9; *p < 0.05 by ANOVA).
Error bars represent SEM.

Leptin Inhibits Firing of Dopamine Neurons in VTA Slices

While the animal studies are consistent with leptin action in VTA neurons, it is possible that the changes in activity are secondary to direct action in the hypothalamus. Experiments were performed to examine if leptin directly influences the electrophysiological responses of dopamine neurons by whole-cell patch-clamp recordings in the VTA of brain slices (Figure 5). Dopamine neurons under electrophysiological analysis were within the boundaries of the VTA and were identified based on their characteristic hyperpolarization-activated (*I*h) current (Johnson and North, 1992; Liu et al., 2005). Spontaneous action potentials were recorded in dopamine neurons under current clamp after their identities were confirmed. The average frequency of action potentials in all tested dopamine neurons was 2.8 ± 0.8 Hz (n = 9). After at least 10 min of stable recording of action potentials, leptin (100 nM, as used by Cowley et al., [2001]) was applied to the recorded dopamine neurons via bath application. Leptin induced a fast and reversible reduction of the frequency of action potentials in dopamine neurons (Figure 5B). The frequency of action potential was reduced to 79.7% ± 6.6% of control in the presence of leptin and 103.8% ± 7.5% of control after the withdrawal of leptin (Figure 5C). The reduction of the frequency of action potentials was significant ($F_{(1,25)}$ = 5.43, $p < 0.05$). The presence of leptin caused a modest, but not significant, hyperpolarization in membrane potential (control, −43.8 ± 1.3 mV; 100 nM leptin, −44.7 ± 1.2 mV; n = 9; $p > 0.05$ by t test). This response suggests that leptin can modify VTA dopamine neuron excitability via direct action that is independent of the hypothalamus.

Conditional *Lepr* Knockdown in the VTA Results in Increased Food Intake and Locomotor Activity

To complement the leptin administration studies, a rat genetic model with reduced *Lepr* expression was developed. Viral-mediated RNA interference (RNAi) was used to generate rats with conditional gene knockdown of *Lepr* specifically in the VTA. Adenoassociated virus (AAV) vectors were engineered to express either a short hairpin RNA (shRNA) targeting *Lepr* mRNA (AAV-shLEPR), or a shRNA that does not have an endogenous mRNA target to serve as a negative control (AAV-shCTRL). This strategy offers both spatial and temporal control of gene knockdown and has been shown to be effective in mouse VTA dopamine neurons (Hommel et al., 2003). Incorporation of an EGFP marker into the viral construct allowed the regions of infection to be accurately identified. Fluorescent microscopy analysis demonstrated that the AAV efficiently infects rat VTA and that a high population of dopamine neurons are infected (Figure 6A). Animals with infection limited to the VTA were chosen for the final data analysis (see Figure S4).

AAV-shLEPR or AAV-shCTRL was administered directly into the VTA of rats, resulting in *Lepr* conditional knockdown ($LEPR^{VTA}$) and control ($CTRL^{VTA}$) animals, respectively. A reduction in *Lepr* mRNA levels in $LEPR^{VTA}$, but not $CTRL^{VTA}$, rats was demonstrated by in situ hybridization analysis of the VTA (Figure 6B). To evaluate changes in behavior, a set of rats was infused bilaterally with either AAV-shLEPR or AAV-shCTRL. Following infusion, food intake and body weight measurements over a 30 day period revealed that $LEPR^{VTA}$ animals increased their food intake compared to $CTRL^{VTA}$ animals (AAV-shLEPR treatment main effect, $F_{(1,20)}$ = 4.26, $p < 0.05$; Figure 6C). The $LEPR^{VTA}$ animals began to show increased feeding between the second and third week following viral delivery, consistent with the time of peak AAV expression and RNAi-mediated knockdown within VTA neurons (Hommel et al., 2003). Despite the increase in food intake, no change was observed in the average body weight of $LEPR^{VTA}$ animals (Figure 6D). In addition, $LEPR^{VTA}$ rats had a 3- to 5-fold increase in ambulatory beam breaks when compared to $CTRL^{VTA}$ rats, with the differences apparent during the dark (active) cycle (Figure 6E) .

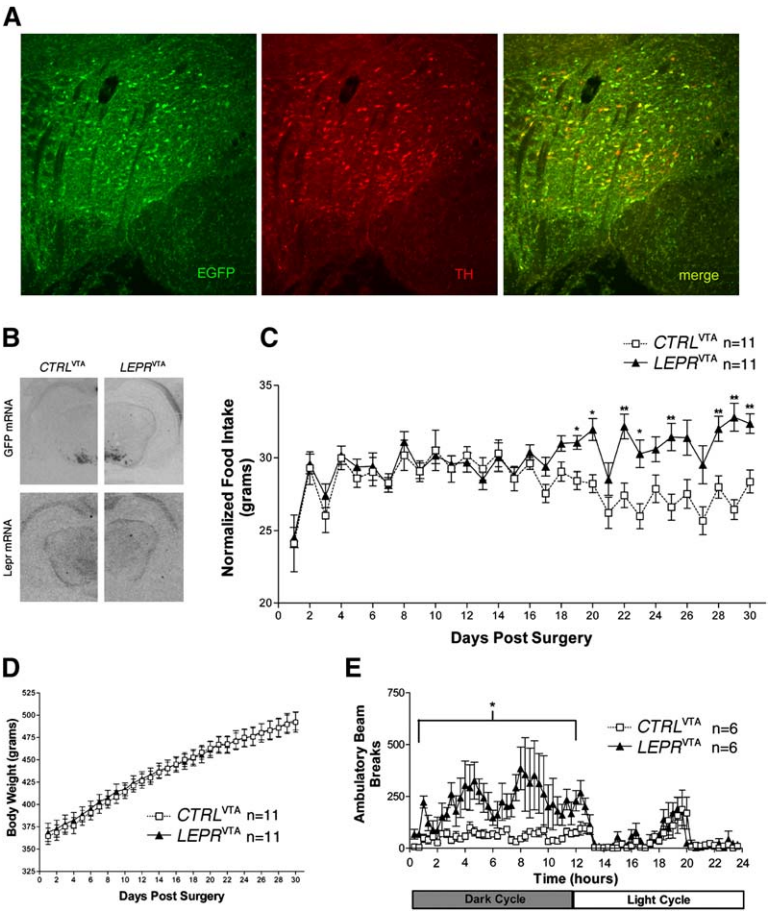

Figure 6. RNAi-Mediated Knockdown of *Lepr* in the VTA Results in Increased Food Intake and Locomotor Activity

(A) Immunofluorescent analysis showing the extent of AAV spread and dopamine neuron infection in the VTA. The left panel shows EGFP expression marking infected neurons, while the middle shows tyrosine hydroxylase (TH). The right panel (merge) demonstrates extensive infection of VTA dopamine neurons.

(B) Representative in situ hybridization of EGFP mRNA in the VTA showing infection and expression from both AAV-shLEPR and AAV-shCTRL viruses (upper panels). In situ hybridization from midbrain tissue showing a reduction in *Lepr* mRNA in $LEPR^{VTA}$ (lower right panel) verses $CTRL^{VTA}$ rats (lower left panel).

(C) Food intake measurements of $CTRL^{VTA}$ (open squares, n = 11) or $LEPR^{VTA}$ rats (closed triangles, n = 11; *p < 0.05; **p < 0.01 by ANOVA followed by post hoc tests).

Total body weight (D), and home-cage ambulatory activity (E), is plotted over time for $CTRL^{VTA}$ (open squares) or $LEPR^{VTA}$ (closed triangles) rats. Home-cage locomotor activity is expressed as number of ambulatory beam breaks and covers 24 hr beginning at the onset of the dark cycle (*p < 0.05 by repeated measures ANOVA during dark cycle).

Error bars represent SEM.

Conditional *Lepr* Knockdown Rats Have Altered Responses to Highly Palatable Food

Following the food intake measurements of the $CTRL^{VTA}$ and $LEPR^{VTA}$ rats, we tested the same animals for responses to a sucrose solution and high-fat food. In the sucrose-sensitivity test, animals were maintained in their home cage and given a choice between two bottles: one bottle contained water and the other bottle a low concentration of sucrose (0.2% w/v) which is not high enough to produce a significant preference in $CTRL^{VTA}$ animals. However, the $LEPR^{VTA}$ rats exhibited increased sensitivity to sucrose, as they consumed 50% more of the 0.2% sucrose solution than water alone (Figure 7A). To assess feeding responses to fat, $LEPR^{VTA}$ and $CTRL^{VTA}$ rats were switched from standard rodent chow to a high-fat rodent chow (45% calories from fat). $LEPR^{VTA}$ rats significantly increased their food intake over the first three days of being presented with a high-fat diet, while $CTRL^{VTA}$ rats did not alter their food intake (in grams) when switched to a high-fat diet. $LEPR^{VTA}$ rats returned to their baseline intake during days 4–7 of the high-fat diet treatment (Figure 7B). This increase in caloric intake over the first 3 days did not result in a significant increase in body weight in the $LEPR^{VTA}$ rats compared to $CTRL^{VTA}$ rats (Figure S5).

Discussion

Based on the expression of *Lepr* in the VTA, we hypothesized that leptin signals directly to midbrain dopamine neurons to regulate energy homeostasis. We tested this hypothesis by administering leptin directly to the VTA or by knocking down *Lepr* in the VTA followed by measuring food intake, body weight, and locomotor activity. Four principal findings emerged: (1) administration of leptin either peripherally or directly to the VTA causes phosphorylation of STAT3 primarily in dopamine neurons; (2) leptin decreases the firing rate of VTA dopamine neurons in vivo and in vitro; (3) delivery of leptin to the VTA decreases food intake but does not alter general locomotor activity; (4) long-term genetic knockdown of *Lepr* in the VTA causes an increase in food intake and dark-cycle locomotor activity but does not alter body weight. Together, the biochemical, cellular, and behavioral data presented indicate that leptin can regulate food intake and locomotion by directly modulating dopamine neuron function in the VTA.

The present data, together with evidence that leptin decreases nucleus accumbens dopamine levels (Krugel et al., 2003), suggest that leptin inhibits the firing of VTA dopamine neurons leading to a decrease in both dopamine release and food intake. This is consistent with the changes in mesolimbic and mesocortical dopamine circuits and feeding behavior following GABA or opioid modulation of the VTA (Badiani et al., 1995; Echo et al., 2002; Mucha and Iversen, 1986; Noel and Gratton, 1995) and work demonstrating that dopamine is critical for the development of obesity in leptin-deficient mice (Szczypka et al., 2000). Previous studies have shown that leptin crosses the blood-brain barrier in most brain

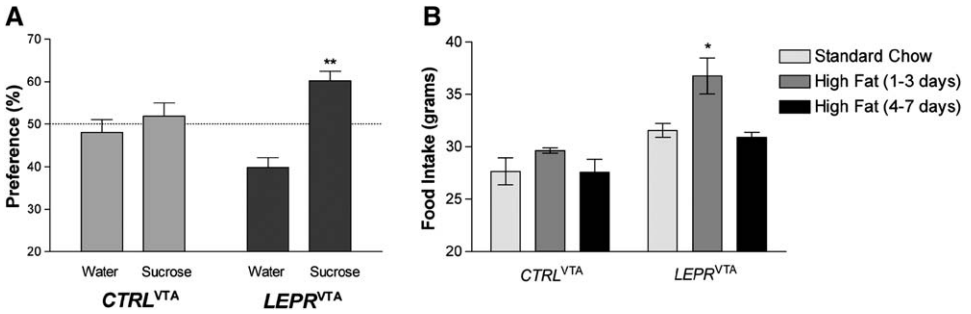

Figure 7. RNAi-Mediated Knockdown of *Lepr* in the VTA Results in Increased Sensitivity to Sucrose and Highly Palatable Food

(A) Preference expressed as percentage of total intake of a 0.2% sucrose solution for both $LEPR^{VTA}$ (n = 8) and $CTRL^{VTA}$ (n = 7) rats in a two-bottle choice paradigm.

(B) Average intake of standard chow and high-fat chow. $LEPR^{VTA}$ rats increased their food intake over the first 3 days when switched to a high-fat chow but returned to baseline intake during the next 4 days.

Error bars represent SEM.

regions, including the midbrain (Banks et al., 2000). In addition, evidence is building for direct action of other peripheral factors, such as insulin and glucose, on dopamine neurons of the midbrain (Figlewicz, 2003; Levin, 2000). Interestingly, recent work suggests that ghrelin can also signal to the VTA to stimulate food intake (Naleid et al., 2005). Therefore, the VTA may represent an additional central detector of peripheral metabolic signals, which responds by appropriately increasing or decreasing food intake via modulation of mesolimbic and mesocortical circuits.

It is striking that $LEPR^{VTA}$ animals, despite increased feeding, maintained normal body weight. Leptin is known to regulate both feeding and metabolism (Friedman and Halaas, 1998; Kamohara et al., 1997), and recent work describes the potential segregation of leptin effects via alternate sites of melanocortin receptor function (Balthasar et al., 2005). In addition, our data support a model whereby leptin effects are segregated by action on different neuroanatomical regions; the VTA may represent a leptin target that modulates motivated behavior and food intake. It has previously been suggested that leptin modulation of conditioned place preference for sucrose and high-fat diets may be mediated by an extrahypothalamic target (Figlewicz et al., 2004; Figlewicz et al., 2001). Our data demonstrate a direct effect of leptin on VTA dopamine neurons and support the previous proposal that leptin modulation of expression of conditioned behavior might occur via dopamine circuits (Figlewicz et al., 2004). Interestingly, a high-fat diet has been shown to decrease conditioned preference for nicotine while also leading to decreased Lepr mRNA in the VTA (Blendy et al., 2005).

It is possible that the VTA-specific knockdown animals maintain normal body weight due to homeostatic metabolic compensation driven by either normal *Lepr* function in the hypothalamus or a separate system compensating for increased caloric intake. The increase in locomotor activity seen in the $LEPR^{VTA}$ animals may itself represent a compensatory response to increased caloric intake. Alternatively, the established role of the VTA in locomotor activity makes it possible that decreases in *Lepr* signaling in the dopamine neurons could lead to an initial increase in locomotor behavior followed by an increase in food intake. However, leptin administration to the VTA resulted in a reduction in feeding without any changes in general locomotor activity, suggesting that a specific effect on feeding is the primary response to increased *Lepr* signaling.

In addition to the increase in consumption of standard rodent chow, the $LEPR^{VTA}$ conditional knockdown rats also showed heightened sensitivity to sucrose and increased consumption of a high-fat diet. This indicates that leptin action on the VTA can also modulate behavioral responses to rewarding or novel food, consistent with proposed roles of the VTA dopamine neurons (Schultz and Dickinson, 2000) and observations of dopamine release in the NAc in response to novel food (Bassareo and Di Chiara, 1999). While our work has focused on a natural behavior (feeding), hormonal regulation of VTA neuronal activity may have consequences for a number of other behavioral responses that are known to be sensitive to the function of dopamine circuits. In particular, metabolic sensing by the VTA neurons may provide a mechanism for the well-described increases in drug sensitivity seen during states of food restriction and the effects of leptin on reversing this sensitivity (Carr, 2002; Shalev et al., 2001). Interestingly, chronic cocaine administration has been shown to alter JAK-STAT signaling in the VTA (Berhow et al., 1996), suggesting that common molecular components mediate metabolic sensing and adaptations to drugs of abuse in VTA dopamine neurons.

As with *Lepr* function in the hypothalamus, a number of questions remain regarding leptin action within the VTA. First, the limited effect of leptin on membrane potential is in contrast to previous reports on hypothalamic neurons (Cowley et al., 2001; Spanswick et al., 1997) and may suggest a distinct mechanism for leptin modulation of excitability within the VTA. Second, it is not clear if the critical *Lepr*-mediated signals are short term, long term, or both. The kinetics of the electrophysiological response following leptin administration indicate that rapid signaling (timescale of minutes) downstream of *Lepr* may be important. However, leptin induced JAK-STAT activation, and the known role for STAT activation in food intake (Bates et al., 2003) supports the premise that long-term transcriptional changes may also play a role. Likewise, recent observations of hypothalamic synaptic rearrangement within 6 hr following leptin and

ghrelin treatment (Pinto et al., 2004) would suggest a potential mode of regulation that may be relevant within mesolimbic and mesocortical circuits.

In summary, the data presented in this paper support a distributed model whereby leptin signals directly to independent brain circuits to generate an overall behavioral response. While previous work has established the hypothalamus as a critical brain region in response to leptin, the inclusion of the VTA as a site of leptin action suggests that peripheral metabolic states are communicated to mesolimbic regions that are integral to the control of motivated behavior. Leptin-mediated modulation of central dopamine circuits provides a novel neural pathway by which changes in leptin levels would lead to adaptive feeding responses. These results also have potential implications for other behaviors, such as drug addiction, where mesolimbic dopamine function is important.

Experimental Procedures

Subjects

Sprague-Dawley rats (Charles River, Kingston, North Carolina), and C57BL/6J mice (Jackson Labs, Bar Harbor, Maine), were used for experiments. Rats were single-housed, whereas mice were housed 4–5 per cage in a colony maintained at constant temperature (23°C) with 12 hr light/dark cycle (lights on from 7:00 AM to 7:00 PM) and ad libitum food and water. Rats weighed between 350 and 400 g at the beginning of the experiments, and mice were 10 weeks of age. All animal protocols were approved by the animal care committees at the University of Texas Southwestern Medical Center and Yale University School of Medicine.

In Situ Hybridization

For the in situ analysis, fresh-frozen rat brains were cryosectioned at 14 μm thickness and mounted onto slides. The sections were then fixed in ice-cold 4% paraformaldehyde for 20 min, dehydrated in an ethanol series, and allowed to air dry. The sections were rehydrated, acetylated for 5 min, dehydrated, and air-dried again. Tissue was then treated differently for fluorescent or radioactive in situ hybridizations.

For fluorescent in situ hybridization, probes were prepared using an in vitro transcription kit with digoxigenin-labeled for *Lepr* (Roche, Basel, Switzerland) and fluorescein-labeled UTP (Molecular Probes, Eugene, Oregon) for TH probes. The hybridization solution (50% formamide, 5× SSC, 5× Denhardts, 250 μg/ml yeast RNA, 0.5 mg/ml salmon testes DNA, 200–300 ng/ml RNA probe) was then added to the slides followed by incubation in humidified chambers at 60°C overnight. After washing and blocking with 5% normal rabbit IgG and 1% blocking reagent (Roche), the *Lepr* probe was first detected by use of a 1:200 anti-digoxigenin antibody coupled to horseradish peroxidase (HRP) (DAKO, Carpinteria, California). The digoxigenin signal was amplified and detected using TSA-direct coupled to Cy3 (Perkin-Elmer, Wellesley, Massachusetts). Hydrogen peroxide treatment (3%, 15 min) was used to eliminate horseradish peroxidase activity. The TH fluorescein-labeled probe was detected with 1:500 rabbit anti-fluorescein HRP (Molecular Probes) followed by amplification with TSA-direct coupled to fluorescein (Perkin-Elmer). The sections were then dehydrated and mounted in DPX (Fluka, Germany).

Radioactive in situ hybridization was performed as previously described (Gold et al., 2002). Hybridization solution (deionized formamide 50%, polyvinyl pyrrolidone 0.7%, ficoll 0.7%, bovine serum albumin 7 mg/ml, denatured salmon sperm DNA 0.33 mg/ml, yeast tRNA 0.15 mg/ml, dithiothreitol 40 μm, and cRNA probe at 1×10^7 cpm/ml) was applied to the slide (120 μl/slide) and covered with Hybrislips (Molecular Probes). Slides were then hybridized overnight at 60°C. Following hybridization, the Hybrislips were removed, and slides were washed twice in 4× saline sodium citrate buffer (SSC) at 60°C for 30 min each. Slides were then RNase treated for 30 min at 45°C followed by two washes in 2× SSC for 10 min, two washes in 0.5× SSC for 10 min, and one wash in 0.1× SSC for 30 min all at 60°C. Slides were then exposed to Biomax MR film (Kodak, Rochester, New York) for 1–2 weeks and developed.

Western Blot Analysis

Mice were food restricted overnight followed by intraperitoneal injections of leptin (3 mg/kg) or vehicle (1× PBS). Leptin was obtained from R&D Systems (Torrance, California) and dissolved according to the manufacturer's protocol. Two hours after injection, brains were rapidly dissected, and the VTA was microdissected using a microscope and a 15 gauge blunt needle. Frozen tissue samples were sonicated in 1% SDS plus 50 mM NaF followed by addition of 2-mercaptoethanol and boiling for 10 min. Small aliquots of the homogenate were retained for protein determination by the DC protein assay method (Bio-Rad, Hercules, California) using bovine serum albumin as a standard. Equal amounts of protein (40 μg) were separated by SDS-polyacrylamide (10%) gel electrophoresis and transferred to PVDF membranes (0.2 μm; Bio-Rad). Membranes were blocked for 60 min in TBST (25 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.1% Tween 20) containing 5% nonfat dry milk and immunoblotted using a 1:1500 dilution of rabbit anti-phospho-STAT3 antibody (Cell Signaling #9131). After washing four times for 20 min in TBST, antibody binding was revealed by incubation with a 1:5000 dilution of goat anti-rabbit HRP-linked IgG (Pierce, Rockford, Illinois) and the SuperSignal West Dura immunoblotting detection system (Pierce). Chemiluminescence was detected by autoradiography using Kodak autoradiography film.

Immunohistochemistry

After cannulation and recovery, rats were infused with 500 ng/side of leptin or vehicle (see below). After 45 min, animals were injected with an overdose of pentobarbital and perfused transcardially with ice-cold 1× PBS for 1 min followed by 4% paraformaldehyde in 1× PBS for 20 min. The brains were postfixed overnight in 4% paraformaldehyde with 1× PBS and then cryoprotected in 1× PBS with 20% glycerol for a minimum of 24 hr. Brains were frozen on dry ice and sectioned at 35 μm intervals and collected in 1× PBS plus 0.05% sodium azide. Sections were pretreated as previously described (Hosoi et al., 2002) and blocked with 3% normal donkey serum in 1× PBS with 0.3% Triton X-100 for 1 hr. The blocking solution was removed and the rabbit anti-phospho-STAT3 (1:500, Cell Signaling Technology #9131, Beverly, Massachusetts) and mouse anti-TH, (1:10,000, Sigma-Aldrich, St. Louis, Missouri) antibodies were diluted in blocking solution and applied to sections. Following overnight incubation, the sections were rinsed three times for 5 min in 1× PBS followed by incubation with fluorescent coupled secondary antibodies (Jackson ImmunoResearch, West Grove, Pennsylvania) in 1× PBS for 2–4 hr. Sections were mounted on slides and dried overnight. Slides were rehydrated for 30 min and washed in 1× PBS three times for 5 min followed by ethanol dehydration and mounting in DPX.

Cannulation Surgery

Rats were allowed to acclimate for 1–2 weeks after arrival before guide cannulae (Plastics One, Roanoke, Virginia) were implanted in the VTA bilaterally. Coordinates for placement of the guide cannula to end 0.8 mm above the injection point in the VTA were 5.7 mm posterior to bregma, 0.75 mm lateral from the midline, 7.8 mm ventral from the surface of the skull (Paxinos and Watson, 2005). Small holes were drilled into the skull and self-tapping stainless steel screws placed to secure the cranioplastic cement (Plastics One) to the skull. Sites for the cannula guides were marked and holes drilled through the skull for the bilateral placement of the 26 gauge stainless steel guides. Once in place, the guides were attached to the skull using cranioplastic cement. When the cement was dry, the animal was removed from the stereotaxic instrument, and sterile obturators (33 gauge; 0.8 mm longer than the guide cannula) were inserted into the guides to prevent them from clogging and to reduce the potential for brain infection. The obturators were checked daily and cleaned as needed.

Microinjection Procedure

After surgery, the rats were allowed to recover for an additional week followed by daily manipulation and habituation to the injection

procedure for 4 days. The injectors were 0.8 mm longer than the guide cannulae. Once the injectors were inserted, the rats were placed into an open cage and allowed to move freely during the infusion. The infusion pumps were operated for 1 min, delivering 0.5 μl of drug or vehicle per side. Leptin was obtained from R&D Systems (Torrance, California) and 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 the sterile obturators replaced.

Feeding Behavior

On the day of the test, the food was removed at 6:30 PM, and the rats were allowed to remain in their home cage until the infusion of either vehicle (1 × PBS) or leptin. Following injection, the rats were returned to their home cage and were allowed full access to regular chow with feeding measurements beginning 30 min later. For long-term studies, food intake and body weight were measured between 4:00 PM and 5:00 PM daily. Standard rodent chow (Harlan Teklad, Indianapolis, Indiana, #7001) containing 12% (by kcal) fat was used. For high-fat diet studies, rodent diet with 45% (by kcal) fat was used (#D12451, Research Diets, New Brunswick, New Jersey).

Locomotor Assay

Rats implanted with bilateral cannulae in the VTA were habituated to the microinjection procedure as they had been previously infused for feeding studies. On the test day, leptin or vehicle (1 × PBS) was delivered 30 min after onset of the dark cycle. Animals were placed in circular locomotor chambers (MED Associates, St. Albans, Vermont) and locomotor activity measured over 2 hr. For the long-term studies, AAV-shCTRL- and AAV-shLEPR-injected animals were habituated to the locomotor room for 4 hr. Home-cage locomotor activity (San Diego Instruments, San Diego, California) was measured by beam breaks over a 24 hr period.

Determination of Cannula Placements

At the completion of the infusion experiments, animals were sacrificed via transcardial infusion of 4% paraformaldehyde. Brain sections were then analyzed by histology via cresyl violet Nissl stain as well as more detailed immunofluorescent analysis to evaluate the exact placement relative to tyrosine hydroxylase staining. Please see Figure S2 for summary analysis and pictures.

Extracellular Single-Unit Recording

Impulse activity of VTA dopamine cells was determined using previously described methods (Henry et al., 1989; Marinelli et al., 2006; Marinelli and White, 2000; Ungless et al., 2004). Briefly, recordings were made from naïve rats, which were anesthetized with chloral hydrate (400 mg/kg, i.p.), and mounted in a stereotaxic apparatus (David Kopf Instruments, Tujunga, California). A lateral tail vein was catheterized to administer additional anesthetic or drugs. Body temperature was monitored by a rectal thermometer (Poly Medica Healthcare Inc., Golden, Colorado) and maintained at 37.0°C (±0.5°C) with a thermostatically controlled heating pad (Fintronics Inc., Orange, Connecticut). A burr hole was drilled in the skull, and the dura mater was retracted from the area overlying the VTA. A glass electrode with a tip diameter of 1–2 μm and filled with a 2 M NaCl solution saturated with 1% fast green dye (Fisher Scientific Co., St Louis, Missouri) was slowly advanced with a hydraulic microdrive (David Kopf Instruments) to the dopamine cell region. The coordinates for the VTA were as follows: 2.8–3.6 mm anterior to lambda, 0.3–0.7 mm lateral from the midline, and 7.8–8.6 mm ventral from the cortical surface (Paxinos and Watson, 2005).

Dopamine cells were identified by anatomical location in the VTA and according to standard physiological criteria (White, 1996). These neurons had (1) a characteristic triphasic (+/−/+) waveform with a wide duration action potential of 2.5–3.5 ms, (2) low spontaneous firing rates of 0.5–10 Hz, and (3) either a slow irregular firing pattern or a slow bursting pattern with decreasing spike amplitude and increasing interspike interval within the burst. During extracellular recording, electrical signals were detected by a high-impedance amplifier (Fintronics Inc.) and monitored by an oscilloscope (Tektronic R5110, Chicago, Illinois) using a window discriminator and an audio-amplifier to detect single-unit events (Grass AM8, Quincy, Massachusetts). An analog-to-digital interface digitized the signal (Digidata 1200 series, Axon Instruments Inc., Foster City, California) to a PC running AxoScope software (Axon Instruments Inc.) that measured impulse activity on-line and stored all data for future analysis. Stored data were then analyzed with a custom-made program from our laboratory (BURST, M.M.) that calculated firing rates (number of spikes/sec: Hz). Neuronal activity of VTA dopamine neurons was determined on 3–4 cells per rat to establish a preleptin baseline activity. Then, saline or leptin (2 mg/kg IV) was administered over 4 min (0.5 mg/kg/min). Dopamine cell activity, during and after saline or leptin administration, was recorded as long as possible in the same cell (15–40 min). When the cell was lost, additional cells were recorded at different time points after the administration of the drug (postsaline or postleptin activity). Each pre- or postleptin cell was recorded for at least 3 min and needed to exhibit stable activity (<5% variation) over at least 2 min to be included in the study. Basal firing rates were 4.61 ± 0.71 for the saline group and 4.77 ± 0.40 for the leptin group. A total of 78 neurons was recorded from 10 rats with the number of neurons for each time bin are as follows: for leptin (47 total neurons from five rats), −100–−30, five neurons; −25–0, 13 neurons; 1–10, nine neurons; 11–20, six neurons; 21–35, seven neurons; 45–90, seven neurons; for saline (31 total neurons from 5 rats), −100–−30, eight neurons; −25–0, seven neurons; 1–10, five neurons; 11–20, three neurons; 21–35, two neurons; 45–90, six neurons. Data are presented as values recorded at different times before (baseline activity) or after saline or leptin administration. All electrode placements were confirmed by passing a 28 μA cathodal current through the electrode. The exact location of the tip of the electrode could then be located via histological analysis of the fast green dye deposition site (see Figure S3 for an example).

In Vitro Recordings in Brain Slices

Brain slices (300 μm) containing the VTA were prepared from 2- to 3-week-old male mice. Animals were anesthetized with Nembutal (80 mg/kg) and then decapitated. The brains were then rapidly removed and immersed in a cold (4°C) oxygenated bath solution (containing 220 mM sucrose, 2.5 mM KCl, 1 mM $CaCl_2$, 6 mM $MgCl_2$, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, and 10 mM glucose [pH 7.3] with NaOH). After being cut on a vibratome and trimmed to contain only the VTA, slices were transferred to a storage chamber where they were constantly perfused with bath solution at 2 ml/min at room temperature. Before recording slices were maintained in a recording chamber at 33°C and perfused continuously with ASCF (bubbled with 5% CO_2 and 95% O_2) containing 124 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 1.23 mM NaH_2PO_4, 26 mM $NaHCO_3$, 10 mM glucose (pH 7.4) with NaOH, for at least 1 hr. Whole-cell current clamp was used to observe spontaneous action potentials in dopamine neurons in the VTA. The pipette solution contained 135 mM K-gluconate, 2 mM $MgCl_2$, 10 mM HEPES, 1.1 mM EGTA, 2 mM Mg-ATP, 10 mM Na_2-phosphocreatine, and 0.3 mM Na_2-GTP (pH 7.3) with KOH. Dopamine neurons in the VTA were identified by presence of a large *I*h current (>100 pA) evoked by hyperpolarizing voltage steps from −50 to −120 mV for 2 s (Johnson and North, 1992; Liu et al., 2005). More than 90% of dopamine neurons in the VTA can be identified with accuracy with this approach (Johnson and North, 1992; Liu et al., 2005). Leptin was applied to the recording chamber via bath application after a stable recording of action potentials was obtained. All data were sampled at 3–10 kHz and filtered at 1–3 kHz with an Apple Macintosh computer using Axograph 4.9 (Axon Instruments). Electrophysiological data were analyzed with Axograph 4.9 (Axon Instruments) and plotted with Igor Pro software (WaveMetrics, Lake Oswego, Oregon).

Design and Construction of shRNA

Hairpin RNA was designed to target specific regions of *Lepr* mRNA. We identified a 24 nucleotide sequence within the coding region of *Lepr* using published criteria (Reynolds et al., 2004). The hairpins were designed such that the antisense strand came before the sense strand during transcription. We synthesized two sets of oligonucleotides (Qiagen) for cloning: shLEPR (top, 5′-*TTTG*AAATCTTTAAATTACCATCATCTTTCGAGATGATGGTAATTTAAAGATTTATTTTT-3′; bottom, 5′-CTAGAAAAATAAATCTTTAAATTACCATCATCTCGAAAGATGATGGTAATTTAAAGATTT-3′), and shCTRL (top, 5′-TTTGTGGAGCCGAGTTTCTAAATTCCGCACCGGAATTTAGAAACCCGGCT

CCACTTTTT-3′; bottom, 5′-CTAGAAAAAGTGGAGCCGGGTTTCTA AATTCCGGTGCGGAATTTAGAAACTCGGCTCCA-3′). The oligonucleotides had BbsI and XbaI overhangs to allow for ligation into the mU6pro plasmid, and all final clones were verified by sequencing. The mU6 promoter, hairpin sequence, and terminator sequences were cut out using ApaI and KpnI sites and ligated into an AAV plasmid designed to coexpress EGFP under the control of an independent RNA polymerase II promoter and terminator (Hommel et al., 2003).

Viral Production and Purification

Viral production was accomplished using a triple-transfection, helper-free method and purified as previously described (Hommel et al., 2003). Briefly, HEK293 cells were cultured in ten 150 × 25 mm cell culture dishes and transfected with pAAV-shRNA, pHelper, and pAAV-RC plasmids (Stratagene) using a standard calcium phosphate method. Cells were collected, pelleted, and resuspended in freezing buffer (0.15 M NaCl and 50 mM Tris [pH 8.0]) 66–70 hr after transfection. After two freeze-thaw cycles to lyse the cells, Benzoase was added (Calbiochem, 50 U/ml, final), and the mixture was incubated at 37°C for 30 min. The lysate was added to a centrifuge tube containing a 15%, 25%, 40%, and 60% iodixanol step gradient. The gradient was spun at 350,000 × g for 60 min at 18°C, the 40% fraction was collected and exchanged with 1× PBS using Amicon BioMax 100K NMWL concentrators. The final purified virus was stored at −80°C. The virus was visualized for purity by silver stain and titered by infection of sodium butyrate-treated HT1080 cells.

Viral Delivery

Rats were anesthetized using pentobarbital and mounted into a stereotaxic apparatus (Kopf). For VTA targeting, Hamilton syringe needles (at 10° angle) 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 μl of purified virus (1–2 × 10^{11} infectious particles/ml) was delivered per side over a 5 min period.

Sucrose Preference

The sucrose preference experiments were done as previously described (Bolanos et al., 2003). Briefly, at the conclusion of the feeding experiments (1 month after the delivery of the virus), animals were habituated to drink from two water bottles for 4 days in their home cages. At approximately 3:00 PM daily, water consumption from each bottle was measured to ensure that there were no baseline differences in bottle preference. Then, the animals were given a 0.2% sucrose solution in one of the bottles. The liquid consumption in each bottle was measured every 24 hr over a period of 2 days, and the position of the bottles was alternated between the 2 days to compensate for bottle-position preference. The percentage of sucrose water consumption over total fluid intake was calculated and expressed as percent sucrose preference. Water stoppers with two bearings were used to prevent leakage.

Data Analysis

Statistical significance was assessed using one-way and factorial design analysis of variance (ANOVA) followed by post-hoc tests. Statistical significance was defined as p value < 0.05.

Supplemental Data

The Supplemental Data for this article can be found online at http://www.neuron.org.cgi/content/full/51/6/801/DC1/.

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