Mesolimbic leptin signaling negatively regulates cocaine-conditioned reward

M Shen, C Jiang, P Liu, F Wang and L Ma

The regulatory mechanisms underlying the response to addictive drugs are complex, and increasing evidence indicates that there is a role for appetite-regulating pathways in substance abuse. Leptin, an important adipose hormone that regulates energy balance and appetite, exerts its physiological functions via leptin receptors. However, the role of leptin signaling in regulating the response to cocaine remains unclear. Here we examined the potential role of leptin signaling in cocaine reward using a conditioned place preference (CPP) procedure. Our results showed that inhibition of leptin signaling by intracerebroventricular infusion of the leptin receptor (LepR) agonist SMLA during cocaine conditioning increased the cocaine-CPP and upregulated the level of dopamine and its metabolites in the nucleus accumbens (NAc). We then selectively knocked down the LepR in the mesolimbic ventral tegmental area (VTA), NAc core and central amygdala (CeA) by injecting AAV-Cre into Leprfl/fl mice. LepR deletion in the VTA increased the dopamine levels in the NAc and enhanced the cocaine-conditioned reward. LepR deletion in the NAc core increased the cocaine-conditioned reward and impaired the effect of the D2-dopamine receptor on cocaine-CPP, whereas LepR deletion in the CeA had no effect on cocaine-CPP but increased the anxiety level of mice. In addition, prior exposure to saccharin increased Lepr mRNA and STAT3 phosphorylation in the NAc and VTA and impaired cocaine-CPP. These results indicate that leptin signaling is critically involved in cocaine-conditioned reward and the regulation of drug reward by a natural reward and that these effects are dependent on mesolimbic LepR.

INTRODUCTION

The hormones of the neuroendocrine system modulate and command perception, cognition and the emotional process. They regulate multiple physiological and pathological states, such as eating and digestion, exploration and survival, and addiction and anxiety. Hormones influence motivation and reward through changing the release of different neurotransmitters in substance abuse and eating disorders. Increasing evidence shows that appetite-regulating hormones such as glucagon-like peptide, ghrelin and leptin are involved in the development processes of substance abuse. Consumption of palatable food and addictive drugs can promote continuous overfeeding or drug-seeking behaviors through eliciting similar hormone-mediated neuro-adaptive changes within brain reward circuitry and stress systems, indicating that there might be shared neuronal circuits or molecular mechanisms in food and addictive rewards.

Leptin, an adipose-derived hormone, affects food intake, body weight, energy balance, emotional behaviour and natural rewarding effects. Leptin inhibits the rewarding effects of food via mesolimbic reward circuits as well as the hypothalamus. Previous work has examined the influence of abnormal systemic leptin levels on addictive behaviors by genetic manipulation or the use of pathological animal models. The systemic injection of leptin and the direct infusion of leptin or the leptin receptor (LepR) agonist into the ventral tegmental area (VTA) attenuates the rewarding effects of cocaine. However, the functional role of leptin signaling within mesolimbic brain regions in regulating the response to addictive drugs still needs to be thoroughly elucidated.

Leptin exerts its biological effects via binding to the functional LepRs, which are widely expressed in the brain. Previous results have shown that specific deletion of LepR in glutamatergic neurons located in the hippocampus and prefrontal cortex causes a depressive-like phenotype, while ablation of the LepR in dopamine neurons results in a robust anxiogenic phenotype, indicating that ablation of the LepR elicits long-term changes in different brain circuits and modulates different behavior phenotypes. However, there may be deficiencies or adaptions during the development of neural circuits in those conditioned knockout mice. Studies examining the effects of acute manipulation of endogenous leptin signaling on drug-reward are lacking.

In this study, we inhibited LepR-mediated signaling in several regions in the mesolimbic system by infusion of the LepR antagonist superactive mouse leptin antagonist (SMLA) or injection of AAV-Cre into the VTA, nucleus accumbens (NAc) or central amygdala (CeA) to selectively deplete LepRs in Leprfl/fl mice. We then investigated the role of leptin signaling in cocaine-conditioned place preference (CPP) and cocaine-induced dopamine release. In addition, the role of mesolimbic LepR signaling on the effect of a natural reward on cocaine-CPP was also assessed.

MATERIALS AND METHODS

Animals and housing

B6 ob/ob mice (strain name: B6.Cg-Leprd+/J) were obtained from the Model Animal Research Center of Nanjing University. Leprfl/fl mice, in which exon 1 of the Lepr gene is floxed, were obtained from The Jackson Laboratory. The regulatory mechanisms underlying the response to addictive drugs are complex, and increasing evidence indicates that there is a role for appetite-regulating pathways in substance abuse. Leptin, an important adipose hormone that regulates energy balance and appetite, exerts its physiological functions via leptin receptors. However, the role of leptin signaling in regulating the response to cocaine remains unclear. Here we examined the potential role of leptin signaling in cocaine reward using a conditioned place preference (CPP) procedure. Our results showed that inhibition of leptin signaling by intracerebroventricular infusion of the leptin receptor (LepR) agonist SMLA during cocaine conditioning increased the cocaine-CPP and upregulated the level of dopamine and its metabolites in the nucleus accumbens (NAc). We then selectively knocked down the LepR in the mesolimbic ventral tegmental area (VTA), NAc core and central amygdala (CeA) by injecting AAV-Cre into Leprfl/fl mice. LepR deletion in the VTA increased the dopamine levels in the NAc and enhanced the cocaine-conditioned reward. LepR deletion in the NAc core increased the cocaine-conditioned reward and impaired the effect of the D2-dopamine receptor on cocaine-CPP, whereas LepR deletion in the CeA had no effect on cocaine-CPP but increased the anxiety level of mice. In addition, prior exposure to saccharin increased Lepr mRNA and STAT3 phosphorylation in the NAc and VTA and impaired cocaine-CPP. These results indicate that leptin signaling is critically involved in cocaine-conditioned reward and the regulation of drug reward by a natural reward and that these effects are dependent on mesolimbic LepR.

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Laboratory (Bar Harbor, ME, USA) (strain name: B6.129P2-Lepr<sup>flox/flox</sup>/J). All mice were bred onto the C57BL/6J background. Lepr<sup>flox/flox</sup> mice and their wild-type (WT) littermates were obtained from self-crossing of Lepr<sup>flox/+</sup> (heterozygous) mice. Mice were housed in groups on a 12 h light/dark cycle with food and water available ad libitum except for in the special case noted below. Genotypes were determined by PCR of mouse tail DNA samples. All animal treatments were strictly in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of the School of Basic Medical Sciences, Fudan University. Male mice, 8–10 weeks old, were used for behavioral tests.

Cannula implantation and microinjection

Mice anesthetized with choral hydrate were placed in a mouse stereotaxic instrument (Stoelting, Kiel, WI, USA) and implanted with cannula guides (Plastics one, Roanoke, VA, USA) over the lateral ventricles, VTA, or NAc core. The cannulas were secured to the mice with dental cement. The intended stereotoxic coordinates were as follows: lateral ventricle: anterior–posterior (AP) −0.2 mm; medial–lateral (ML) ± 1.0 mm; dorsal–ventral (DV) −2.2 mm; VTA: AP −3.2 mm; ML ± 0.5 mm; DV −4.4 mm; and NAc core: AP +1.4 mm; ML ± 1.2 mm; DV −4.3 mm. All mice were given at least 7 days to recover before behavioral experiments.

SMLA (BioSource, San Diego, CA, USA) mimics the Asp-23 mutation of leptin, which exhibits a high affinity for the leptin receptor and antagonistic activity in vitro and in vivo. SMLA was dissolved in artificial cerebrospinal fluid (ACSF) (126 mM NaCl, 26 mM NaHCO3, 1.2 mM NaH2PO4, 3 mM KCl, 2.4 mM CaCl2, 1.3 mM MgCl2, 10 mM d-glucose) to 250 ng μl<sup>−1</sup>, and intracerebroventricular (i.c.v.) administration of SMLA through the cannula was performed at a volume of 2 μl at a slow rate (0.2 μl min<sup>−1</sup>). Mice received microinjection of SMLA (500 ng) or ACSF as the vehicle (2 μl) 30 min before or after cocaine conditioning. D2-dopamine receptor (D2R) agonist bromocriptine (Tocris Bioscience, Bristol, UK) was dissolved in ACSF to 250 ng μl<sup>−1</sup>, and mice received a bilateral microinjection of 2 μl bromocriptine solution into the NAc core at a slow rate (0.2 μl min<sup>−1</sup>) 30 min before cocaine conditioning.

Viral infection

Male adult Lepr<sup>flox/flox</sup> mice and their WT littermates anesthetized with choral hydrate were placed in a mouse stereotaxic instrument. Microinjections were performed using custom-made injection needles (33-gauge) connected to a 10 μl Hamilton syringe. Each brain nucleus was injected with 0.5 μl of purified and concentrated AAV2/5 (5 × 10<sup>12</sup> IU ml<sup>−1</sup>) encoding CAG-eGFP-T2A-Cre at a slow injection rate (0.1 μl min<sup>−1</sup>). The intended stereotoxic coordinates were as follows: NAc core: AP +1.4 mm; ML ± 1.2 mm; DV −4.3 mm; VTA: AP −3.2 mm; ML ± 0.5 mm; DV −4.4 mm; and D2R agonist bromocriptine solution into the NAc core at a slow rate (0.2 μl min<sup>−1</sup>) 30 min before cocaine conditioning.

High-performance liquid chromatography

The levels of monoamine neurotransmitters, including norepinephrine (NA), dopamine (DA) and 5-hydroxytryptamine (5-HT), and their respective metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), and 5-hydroxyindoleacetic acid (5-HIAA), were detected 30 min after cocaine (15 mg kg<sup>−1</sup>) injection, using high-performance liquid chromatography (HPLC) as previously described. Within 5 min of the cocaine injection, the NAc and amygdala of mice were dissected in iced phosphate-buffered saline by a vibratome (Microm HM 650V, Thermo Fisher Scientific, Waltham, MA, USA), according to the stereotoxic coordinates listed below: NAc: from Bregma 1.54 to 0.54 mm and amygdala (AMG): from Bregma −1.06 to −2.06 mm. The NAc and AMG were then homogenized in 100 μl ice-cold 0.1 M perchloric acid containing 10 μM ascorbic acid, 0.1 μM EDTA disodium salt and 0.02 μM 3,4-dihydroxybenzylamine. The homogenates were centrifuged at 20 000 g for 10 min at 4 °C, and the supernatants were collected for analysis. An HPLC system with electrochemical detection (UltiMate 3000 system; Thermo Fisher Scientific) was used, and the supernatants were injected onto an Acclaim C18 column (2.2 μm, 2 × 100 mm; Thermo Fisher Scientific) at 38 °C. Separations were performed at a flow rate of 0.2 ml min<sup>−1</sup> using a mobile phase of phosphate buffer, containing 0.05 μM EDTA, 1.7 mM orthosilicic acid, 90 mM Na2HPO4, 50 mM citric acid and 5% acetonitrile. The data were collected and analyzed by Chromleon chromatography workstation (Thermo Fisher Scientific).

Cocaine- or palatable-food-induced CPP

CPP induced by cocaine hydrochloride (Qinghai Pharmaceutical Firm, Qinghai, China) was blindly performed by an investigator using a two-chamber apparatus (Med-Associates, St Albans, VT, USA) with distinct tactile environments to maximize contextual differences. One chamber of the box had a wire mesh floor, while the other chamber had a grid rod floor. A manual guillotine door (7 × 5 cm) separated the two chambers. On Day 1, mice were placed in one of the chambers and allowed to freely explore the entire apparatus for 20 min (pre-test). The mice staying in one chamber for more than 13 min were excluded from the experiment. Mice were randomized into two groups by tossing a coin to receive vehicle or drugs. On days 2, 3 and 4, mice were given an intraperitoneal injection of cocaine (15 mg kg<sup>−1</sup>, i.p.) in the morning and confined to one of the chambers (drug-paired) for 30 min, and in the afternoon, they received an i.p. injection of saline (an equivalent volume to that of cocaine) and were confined to the other chamber for 30 min (conditioning). The distance the mice traveled within the 30 min was recorded. On Day 5, mice were allowed to freely explore the entire apparatus for 20 min (test). The time spent in each chamber was recorded during the pre-test and test sessions. The CPP score was defined as the time (in seconds) spent in the cocaine-paired chamber minus the time spent in the saline-paired chamber. All mice used in the cocaine-CPP were satiated.

CPP induced by palatable food was assessed using an approach similar to that previously reported. were randomized into two groups by tossing a coin to receive vehicle or drugs. On days 2, 3 and 4, mice were given an intraperitoneal injection of cocaine (15 mg kg<sup>−1</sup>, i.p.) in the morning and confined to one of the chambers (drug-paired) for 30 min, and in the afternoon, they received an i.p. injection of saline (an equivalent volume to that of cocaine) and were confined to the other chamber for 30 min (conditioning). The distance the mice traveled within the 30 min was recorded. On Day 5, mice were allowed to freely explore the entire apparatus for 20 min (test). The time spent in each chamber was recorded during the pre-test and test sessions. The CPP score was defined as the time (in seconds) spent in the cocaine-paired chamber minus the time spent in the saline-paired chamber. All mice used in the cocaine-CPP were satiated.

Sacharin operant task

Each operant behavior apparatus (21.6 cm length × 17.8 cm width × 12.7 cm height, Med-Associates) was equipped with a dim light source and a nose-poke hole (3.8 cm in diameter, 0.64 cm deep, 1.0 cm from the grid floor, Med-Associates) equipped with infrared photo-beams connected to a computer. In the center of the wall was a trough situated 2 cm from the grid floor from which liquid (0.1% saccharin w/v) or water was delivered when the photo-beam of the nose-poke hole was interrupted for at least 500 ms. A fixed-ratio 1 reinforcement schedule was initially applied for 12 consecutive days; that is, one nose-poke resulted in one delivery of liquid. Mice were divided into three groups by generating a random digit: water, acute saccharin and chronic saccharin, and each group of mice were confined to the apparatus for 1 h test daily. The water group received a delivery of water after a nose-poke during the 1-h session for 12 days; the mice in the acute saccharin group received a delivery of water after a nose-poke for 11 days and, on Day 12, received a delivery of 0.1% saccharin as a reward after a nose-poke; the chronic saccharin group received a delivery of 0.1% saccharin after a nose-poke for 12 days. Cocaine CPP was blindly performed by an investigator.

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Statistical analysis
Seven to twelve mice per group were used for behavior tests, and 4–6 mice per group were used for HPLC and biochemistry studies. All data are presented as the mean ± s.e.m. Student’s t-test, two-way analysis of variance (ANOVA), or two-way repeated-measures analysis of variance (RM ANOVA) was used for statistical analysis. Tukey’s post hoc analysis was subsequently performed after a two-way ANOVA or two-way RM ANOVA.

*P < 0.05, **P < 0.01 and ***P < 0.001.

RESULTS

Leptin signaling in the brain negatively regulates cocaine-conditioned reward and cocaine-induced dopamine release in the NAc

To explore the potential role of the endogenous leptin system in regulating drug reward, we examined cocaine (15 mg kg⁻¹ i.p.)-induced CPP in ob/ob mice (systemic knockout of leptin) and their WT littermates (Figure 1a). The WT and ob/ob mice were given an injection of cocaine and trained to associate the drug with one chamber daily for 3 days. In the test session, both groups showed significant preference for the cocaine-paired chamber. ob/ob mice spent significantly more time in the cocaine-paired chamber compared with their WT littermates (two-way ANOVA, treatment × genotype: F(1,17) = 4.240, P = 0.055. WT vs ob/ob within test, P = 0.009; Figure 1b). Leptin exerts its biological effects via activating the functional LepRs, which are widely expressed in the central nervous system.24 To assess the role of LepR signaling in the central nervous system in cocaine-conditioned reward, we tested the effects of i.c.v. infusion of a LepR antagonist, SMLA, on the acquisition of cocaine-CPP. Adult C57 WT mice received microinjection of vehicle or SMLA 30 min before or after the cocaine-context-conditioning period (Figure 1c and Supplementary Figure S1A). The SMLA-treated mice spent more time in the cocaine-paired chamber than the vehicle-treated individuals (Figure 1d: two-way ANOVA, treatment × genotype: F(1, 19) = 1.572, P = 0.218. Vehicle vs SMLA within test, P = 0.019; S1B: treatment × genotype: F(1, 14) = 1.684, P = 0.215. Vehicle vs SMLA within test, P = 0.022; Figure 1d and Supplementary Figure S1B). The cocaine-induced monoamine neurotransmitter levels in the NAc after SMLA i.c.v. administration were examined. The SMLA-treated mice exhibited significantly increased levels of dopamine and its metabolites in the NAc (Student’s t-test, DA: t(7) = 4.0, P = 0.0023; DOPAC: t(7) = 2.875, P = 0.0238; HVA: t(7) = 3.787, P = 0.0068; Figure 1e), while the concentrations of norepinephrine (NE), 5-hydroxytryptamine (5-HT) and its metabolites 5-hydroxyindoleacetic acid (5-HIAA) (NE: t(7) = 0.4596, P = 0.6597; 5-HT: t(7) = 1.096, P = 0.3120; 5-HIAA: t(7) = 1.049, P = 0.3289) in the NAc were not changed (Supplementary Figure S2A). These results indicate that leptin signaling negatively regulates cocaine-conditioned reward and affects mesolimbic dopamine system.

Leptin signaling in the VTA negatively regulates cocaine-conditioned reward

Addictive drugs are thought to activate the reward circuitry in the VTA that projects to the NAc, amygdala, hippocampus and prefrontal cortex.25 To assess the effects of endogenous LepRs in the mesolimbic system on cocaine reward-associated behaviors, we bilaterally injected AAV-CAG-eGFP-T2A-Cre into the VTA of Lepflox/flox mice and their WT littermates 14 days before behavior tests (Figure 2a), and LepR protein expression levels were assessed by immunostaining (Figure 2b). AAV-CAG-eGFP-T2A-Cre injection significantly reduced the number of GFP and LepR-double positive cells in the VTA of Lepflox/flox mice compared with that in the WT littermates (Student’s t-test, t(9) = 4.033, P = 0.0030; Figure 2c and Supplementary Figure S3A), indicating a marked knockdown of endogenous LepRs in the VTA. The cocaine-induced monoamine neurotransmitter levels in the downstream targets of the VTA, including the NAC and AMG, were examined. We found that the AAV-Cre-injected Lepflox/flox mice exhibited increased levels of dopamine in the NAc (Student’s t-test, DA: t(10) = 2.307, P = 0.0438; DOPAC: t(10) = 2.433, P = 0.0347; HVA: t(10) = 1.582, P = 0.1444; NE: t(10) = 0.4968, P = 0.6301; 5-HT: t(10) = 0.1010, P = 0.9216; 5-HIAA: t(10) = 0.6076, P = 0.5570; Figure 2d left panel and Supplementary Figure S2B), while there was no significant difference in the AMG compared with that in WT littermates (Student’s t-test, DA: t(10) = 0.5371, P = 0.6029; DOPAC: t(10) = 0.4601, P = 0.6553; HVA: t(10) = 0.8738, P = 0.4027; NE: t(10) = 0.6575, P = 0.5257; 5-HT: t(10) = 0.1710, P = 0.8676; 5-HIAA: t(10) = 2.156, P = 0.0565; Figure 2d right panel and S2C), suggesting the knockdown of LepRs in the VTA might lead to impaired function of dopaminergic axons innervating NAC. Lepflox/flox mice spent significantly more time in the palatable food-paired chamber than the WT individuals (two-way ANOVA, treatment × genotype: F(1, 16) = 1.959, P = 0.181. WT vs Lepflox/flox within test, P = 0.047) in the test session (Supplementary Figures S1C and D) after five sessions of conditioning, which was consistent with the previous studies that leptin inhibited food reward through its effects on the mesolimbic reward circuitry.26-27

We then evaluated the effects of LepR knockdown in the VTA on cocaine-CPP. After 3 days of cocaine conditioning, both groups showed a preference for the cocaine-paired chamber, while Lepflox/flox mice spent more time in the cocaine-paired chamber than the WT individuals (two-way ANOVA, treatment × genotype: F(1, 17) = 2.739, P = 0.119. WT vs Lepflox/flox within test, P = 0.032; Figure 2e). There was no significant difference between AAV-Cre-injected Lepflox/flox and WT mice in the time spent in the open arms of the elevated plus maze test (Student’s t-test, t(25) = 1.713, P = 0.0991; Figure 2f). In the open-field test, there was no significant difference between Lepflox/flox mice and their WT littermates in the time spent in the center zone, the entries into center, or the total distance travelled (Student’s t-test, time: t(22) = 1.696, P = 0.1040; entries: t(22) = 0.1297, P = 0.8980; total distance travelled: t(25) = 0.4079, P = 0.6869; Figure 2g, Supplementary Figures S4A and B). These data suggest that leptin signaling in the VTA might decrease the dopamine level in the NAC and negatively regulate cocaine-conditioned reward without affecting the anxiety level.

Leptin signaling in the NAC core negatively regulates cocaine-conditioned reward and is critical for the function of the D2R

To assess the effect of leptin signaling in the downstream brain regions of the VTA on cocaine-CPP, we bilaterally injected AAV-CAG-eGFP-T2A-Cre into the NAC core of Lepflox/flox mice and their WT littermates (Figure 3a), and the loss of the LepR in the NAC was evaluated by immunostaining (Student’s t-test, t(15) = 6.943, P < 0.001; Figure 3b and Supplementary Figure S3B). Lepflox/flox mice injected with virus spent more time in the cocaine-paired chamber than their WT littermates (two-way ANOVA, treatment × genotype: F(1, 14) = 2.671, P = 0.124. WT vs Lepflox/flox within test, P = 0.040; Figure 3c). It has been reported that leptin signaling increases D2R activity in the striatum.28 To assess whether NAC leptin signaling is involved in the D2R-dependent pathway, we injected the D2R agonist bromocriptine into the NAC core of AAV-Cre-infected mice 30 min before each conditioning session. The results showed that D2R activation eliminated cocaine-CPP in WT mice but not in Lepflox/flox mice (two-way ANOVA, treatment × genotype: F(1, 17) = 42.617, P < 0.001. WT vs Lepflox/flox within test, P < 0.001) in the test session (Figure 3d), suggesting that LepRs might be essential for the inhibitory effects of cocaine-CPP induced by D2R-positive neurons of the NAC.

The time spent in the open arms in the elevated plus maze experiments (Student’s t-test, time in the open arms: t(16) = 0.4077, P = 0.6889; Figure 3e) and the time in the center zone, the entries into center, and the total distance travelled in the
open-field test also showed no significant differences between AAV-Cre-injected **Lepr**<sup>fl</sup><sup>ox/</sup><sup>ox</sup> mice and their WT littermates (time in the center zone: $t(18) = 0.5111, P = 0.6155$; entries: $t(18) = 0.4255, P = 0.6755$; total distance travelled: $t(18) = 0.1627, P = 0.8726$; Figure 3f, Supplementary Figures S4C and D), suggesting that leptin signaling in the NAc negatively regulates cocaine-conditioned reward and D2R function while having no effects on anxiety or locomotor activity.
Figure 2. Leptin signaling in the VTA negatively regulates cocaine-conditioned reward and dopamine levels in the NAc. (a) Schematic drawing of the AAV-CAG-EGFP-T2A-Cre (left) and the experimental schedule of immunohistochemistry (IHC) and behavior tests after virus injection (right). (b) Representative images of the AAV-CAG-EGFP-T2A-Cre-infected VTA from Lepr\textsuperscript{floxed/floxed} mice and WT littermates. EGFP: green; LepR: red; arrows indicate the EGFP\textsuperscript{+} cells. Scale bar, 100 μm (low-magnification images) and 20 μm (high-magnification images). (c) Quantification of the percentage of LepR-positive cells in the EGFP\textsuperscript{+} populations in the VTA of virus-infected Lepr\textsuperscript{floxed/floxed} mice and WT littermates. (d) Concentration of cocaine-induced monoamine neurotransmitters in the NAC and AMG. (e) Quantification of the place preference scores of AAV-Cre-injected Lepr\textsuperscript{floxed/floxed} and WT mice in the pre-test and test sessions. (f) Time spent in the open arms of the elevated plus maze (test in the Lepr\textsuperscript{floxed/floxed} and WT mice. (g) Time in the center zone of the open-field test in the Lepr\textsuperscript{floxed/floxed} and WT mice. The data are presented as the mean ± s.e.m. *P < 0.05, **P < 0.01. AMG, amygdala; NAc, nucleus accumbens; VTA, ventral tegmental area; WT, wild-type.
Leptin signaling in the CeA is involved in anxiety but not cocaine-conditioned reward.

The CeA, which mediates stress-related processes, is also innervated by VTA transmission. Leprfl/fl mice and their WT littermates received bilateral infusion of AAV-CAG-EGFP-T2A-Cre into the CeA (Figure 4a), and the knockdown efficiency of the LepR in the CeA was evaluated by immunostaining (Student’s t-test, t(13) = 9.665, P < 0.001; Figure 4b). In the cocaine-CPP test, there was no significant difference in the preference scores between Leprfl/fl mice and their WT littermates (two-way ANOVA, treatment × genotype: F(1, 14) = 0.00187, P = 0.966. WT vs Leprfl/fl within test, P = 0.905; Figure 4c).

Figure 3. Leptin signaling in NAc Core negatively regulates cocaine-conditioned reward and is essential for D2R function. (a) Representative images of the AAV-CAG-EGFP-T2A-Cre-infected NAc from Leprfl/fl mice and WT littermates. (b) Quantification of the number of LepR-positive cells in the EGFP+ cells in the NAc core of virus-infected Leprfl/fl mice and WT littermates. EGFP: green; LepR: red; arrows indicate the EGFP+ cells. Scale bar, 100 μm (low-magnification images) and 20 μm (high-magnification images). (c) Quantification of the place preference scores of virus-infected Leprfl/fl and WT mice in the pre-test and test sessions. (d) Schematic of the experimental schedule for cocaine-CPP after cannula implantation (upper panel). Mice received bilateral infusion of the D2R agonist bromocriptine (500 ng) into the NAc core 30 min before cocaine conditioning, and the quantification of the place preference scores in the pre-test and test sessions is shown below. (e) Time spent in the open arms of the elevated plus maze test in Leprfl/fl and WT mice. The data are presented as the mean ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001. CPP, conditioned preference place; D2R, D2-dopamine receptor; LepR, leptin receptor; NAc, nucleus accumbens; WT, wild-type.

Leptin signaling in the CeA is involved in anxiety but not cocaine-conditioned reward. The CeA, which mediates stress-related processes, is also innervated by VTA transmission. Leprfl/fl mice and their WT littermates received bilateral infusion of AAV-CAG-EGFP-T2A-Cre into the CeA (Figure 4a), and the knockdown efficiency of the LepR in the CeA was evaluated by immunostaining (Student’s t-test, t(13) = 9.665, P < 0.001; Figure 4b). In the cocaine-CPP test, there was no significant difference in the preference scores between Leprfl/fl mice and their WT littermates (two-way ANOVA, treatment × genotype: F(1, 14) = 0.00187, P = 0.966. WT vs Leprfl/fl within test, P = 0.905; Figure 4c).
In the elevated plus maze test, the Lepr^floxflox mice injected with AAV-CAG-eGFP-T2A-Cre in the CeA spent significantly less time in the open arms than the WT littermates (Student’s t-test, t(15) = 3.441, P = 0.0036; Figure 4d). In the open-field test, Lepr^floxflox mice spent significantly less time in the center zone (t(15) = 2.108, P = 0.0536; Supplementary Figure S4E). Although the total distance travelled was not different between the two groups (t(14) = 0.5866, P = 0.5668; Supplementary Figure S4F). These data suggest that leptin signaling in the CeA is not essential for cocaine-conditioned reward, while it is involved in the regulation of the negative emotional state.
Leptin signaling in cocaine-conditioned reward

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Figure 5. Upregulated leptin signaling reduces cocaine-CPP. (a, b) Mice received injection of saline or leptin (1 mg kg⁻¹ i.p.) 30 min before conditioning, and the quantification of the place preference scores in the pre-test and test sessions is shown. (c) Schematic of the saccharin reward experimental schedule. Three groups of mice were confined to the apparatus for a 1-h operant task for 12 days to receive water (W) or saccharin daily (CS) or saccharin only on the last session (AS). The mice were decapitated 24 h after the last session. (d, e) The levels of LepR mRNA (d), pSTAT3 protein and total STAT3 (e) in the NAc and VTA were detected. Images are representative of similar results from independent individuals. (f) Schematic of the operant task and cocaine-CPP schedule. Three groups of mice (W, AS and CS) performed a 1-h operant task for 12 days, and 24 h after the last session, each mouse received cocaine-context conditioning learning. Place preference was examined 24 h later. (g) Quantification of the place preference scores of the three groups of mice in the pre-test and test sessions. (h) Schematic of the modified cocaine-CPP schedule. SMLA (500 ng) or vehicle was infused into the NAc or VTA 30 min after saccharin administration, and the mice received cocaine-context conditioning 24 h later. (i, j) Quantification of the place preference scores of mice receiving SMLA (500 ng) infusion into the NAc (i) or VTA (j) after saccharin administration in the pre-test and test sessions. The data are presented as the mean ± s.e.m. *P < 0.05, **P < 0.01. CPP, conditioned place preference; NAc, nucleus accumbens; SMLA, superactive mouse leptin antagonist; VTA, ventral tegmental area.

Upregulation of leptin signaling in the VTA or NAc inhibits cocaine-conditioned reward

Because downregulation of leptin signaling increased cocaine-CPP, we then assessed whether upregulation of leptin signaling might have an opposite effect. Mice intraperitoneally injected with leptin during the cocaine-context conditioning sessions (Figure 5a) showed a decreased preference score (two-way ANOVA, treatment × genotype: F(1, 17) = 4.684, P = 0.038. Saline vs leptin within test, P = 0.044; Figure 5b), suggesting that the activation of LepR decreased cocaine-CPP, which was consistent with the previous result that injection of exogenous leptin in the test session inhibited the establishment of cocaine-CPP in mice.15

We then measured the mesolimbic LepR mRNA and phosphorylated STAT3 levels in mice receiving saccharin to investigate whether palatable food reward affects mesolimbic LepR signaling. WT mice were divided into three groups, assigned to water (W), acute saccharin (AS) or chronic saccharin (CS). Each group of mice was daily confined to the apparatus for 1-h to assess water or saccharin intake (Figure 5c). The levels of LepR mRNA and pSTAT3 protein in the NAc and VTA were assessed 24 h after the last saccharin or water exposure. Saccharin administration significantly increased LepR mRNA levels in the NAc (Student’s t-test, AS: t(8) = 5.943, P = 0.0003; CS: t(8) = 2.375, P = 0.0449 vs W) and VTA (AS: t(8) = 1.811, P = 0.1077; CS: t(8) = 2.406, P = 0.0428 vs W; Figure 5d), and both acute and chronic saccharin administration significantly induced activation of leptin signaling in the NAc and VTA as the level of pSTAT3 protein increased when compared with that of control (Student’s t-test, NAc: AS: t(8) = 2.280, P = 0.0486; CS: t(10) = 2.572, P = 0.0278 vs W; VTA: AS: t(7) = 2.248, P = 0.0418; CS: t(7) = 4.320, P = 0.0035 vs W; Figure 5e). These data demonstrated that saccharin exposure upregulated leptin signaling in the NAc and VTA. Parallel with the upregulation of leptin signaling, cocaine-CPP was nearly abolished in mice from the AS and CS groups (two-way ANOVA, AS: treatment × group: F(1, 17) = 2.499, P = 0.129. AS vs W within test, P = 0.026; CS: treatment × group: F(1, 17) = 6.613, P = 0.015. CS vs W within test, P = 0.001; Figures 5f and g). To assess whether the upregulation of LepR signaling in the
Leptin signaling into the VTA attenuates the rewarding effects of cocaine and knockdown of striatal D2Rs accelerates the development of addiction-like reward deficits in obese rats. Mice lacking leptin systemically show decreased D2R binding in the striatum and increased dopamine activity in reward-related brain regions including the NAc and activation of the LepR inhibits food intake via the D2R indirect pathway. These previous results imply that LepRs are involved in the effect of D2R activation. Here we found that impaired cocaine-CPP upon D2R activation in the NAc was eliminated when LepRs in the NAc core were deleted, which suggests that LepRs are required for the D2R-induced inhibitory effects of cocaine-CPP. The function of leptin signaling in the D1R- or D2R-positive neurons in the NAc will be assessed in the future using Cre-expressing mice and might help elucidate the mechanism of leptin signaling affecting cocaine-CPP.

It has been shown that some compartments of amygdala, especially CeA, code reward-related value and affective significance to regulate reward learning and addiction. LepR-positive neurons innervate cocaine- and amphetamine-regulated transcript neurons in CeA, and intra-amygdala infusion of leptin facilitates the extinction of conditioned fear responses. Here we found that specific deletion of LepR in the CeA of adult mice increased the anxiety level while had no effect on the cocaine-CPP, suggesting that leptin signaling in the CeA might be involved in the affective evaluation, whereas is not essential to the cocaine-conditioned rewards. Chronic exposure to high-caloric food induces obesity and reduces protein levels of D2R, CREB as well as D2R mRNA in the striatum, and also induced functional synaptic changes associated with addictive behavior, indicating food reward changes the homeostasis of reward system. Consumption of palatable food including sweet or fatty foods accelerates habitual control of behavior dependent on activation of the dorsolateral striatum, and enhances cocaine-induced locomotor sensitization which is associated with increased motivation for rewards and reward paired cues. Recent work showed that cocaine, morphine and chocolate of the same valence activate largely overlapping neural ensembles in the NAc. We found that either acute or chronic acquisition of saccharin impaired cocaine-CPP, and significantly upregulated the leptin signaling in the VTA and NAc. Inhibition of the upregulated leptin signaling by SMLA rescued the deficit of cocaine-CPP induced by saccharin reward. These results support the notion that leptin signaling is a negative regulator in the effect of addictive drugs and might also to be involved in mediating the inhibitory effect of natural reward upon drug reward.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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