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# Selective Deletion of the Leptin Receptor in Dopamine Neurons Produces Anxiogenic-like Behavior and Increases Dopaminergic Activity in Amygdala

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## Abstract

Leptin receptors (Lepr) are expressed on midbrain dopamine neurons. However, the specific role of Lepr signaling in dopamine neurons remains to be clarified. In the present study, we generated a line of conditional knockout mice lacking functional leptin receptors selectively on dopamine neurons ($Lepr^{DAT\text{-}Cre}$). These mice exhibit normal body weight and feeding. Behaviorally, $Lepr^{DAT\text{-}Cre}$ mice display an anxiogenic-like phenotype in the elevated plus-maze, light-dark box, social interaction and novelty-suppressed feeding tests. Depression-related behaviors in the chronic stress-induced anhedonia, forced swim and tail-suspension tests were not affected by deletion of Lepr in dopamine neurons. *In vivo* electrophysiological recordings of dopamine neurons in the ventral tegmental area (VTA) revealed an increase in burst firing in $Lepr^{DAT\text{-}Cre}$ mice. Moreover, blockade of D1-dependent dopamine transmission in the central amygdala by local microinjection of the D1 antagonist SCH23390 attenuated the anxiogenic phenotype of $Lepr^{DAT\text{-}Cre}$ mice. These findings suggest that leptin receptor signaling in midbrain dopamine neurons has a crucial role for the expression of anxiety and for the dopamine modulation of amygdala function.

### Keywords

Leptin receptor; dopamine neurons; feeding; anxiety; ventral tegmental area; central amygdala



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## Introduction

The adipocyte-derived hormone leptin is a pleiotropic hormone that affects multiple physiological processes including appetite, body weight, neuroendocrine function and emotional behaviors[1–6]. Leptin exerts its effects by acting on the full-length functional leptin receptor (Lepr), which is expressed in various brain regions[7]. Whether the different functional roles of leptin are mediated by discrete neuronal populations, however, remains to be characterized. A great deal of attention has focused on the Lepr-containing neurons within the subdivisions of the hypothalamus[8–11] and suggested that the hypothalamic targets are essential for the regulation of leptin action on food intake and body weight. The cell populations associated with limbic functions of leptin, however, remain to be clarified. Recent studies suggest that midbrain dopamine neurons are direct targets for leptin[7, 12–15].

Midbrain dopamine neurons of the ventral tegmental area (VTA), projecting to limbic regions including the amygdala, nucleus accumbens and prefrontal cortex, are important neural substrates for rewarding and emotional behaviors[16–23]. Previous studies have demonstrated that leptin modulates the mesolimbic dopamine system at multiple levels including tyrosine hydroxylase (the rate-limiting enzyme in dopamine biosynthesis), vesicular somatodendritic stores, neuronal excitability, dopamine transporter and dopamine release in wild-type rats or leptin-deficient ob/ob mice, although the results across studies have not always been consistent[11, 13, 14, 24–26]. Moreover, it has been reported that administration of leptin into the VTA decreases normal feeding, and adeno-associated virus (AAV)-mediated Lepr knockdown in the VTA increases food intake and the sensitivity to highly palatable foods[14, 27]. However, the specific functional role of Lepr signaling in dopamine neurons in appetite control and energy homeostasis remains unclear. On the other hand, leptin has been found to regulate depression- and anxiety-related behaviors in rats or mice[28–31]. Neuronal circuits that mediate mood-regulating effects of leptin are currently unknown. Given the important role of the mesolimbic dopamine system in mood, motivation and responses to rewarding stimuli, we hypothesized that midbrain dopamine neurons mediate the actions of leptin on feeding and emotional behaviors. To assess the functional role of Lepr in dopamine neurons, we utilized the Cre/loxP system and generated a line of conditional knockout mice that lack Lepr selectively on dopamine neurons. Using this mouse model, we examined the effects of ablation of Lepr in dopamine neurons on feeding, anxiety- and depression-related behaviors, and further characterized possible neural mechanisms underlying behavioral changes.

## Materials and Methods

### Animals

Mice were housed on a 12 h light/12 h dark cycle (lights on at 7:00 h) with *ad libitum* access to food and water. All procedures were approved by the Institutional Animal Care and Use Committee and carried out in accordance with the National Institutes of Health Guide.

## Generation and characterization of mice lacking Lepr on dopamine neurons

**Generation of mice lacking Lepr on dopamine neurons—**To generate conditional knockout mice lacking Lepr in dopamine neurons, Lepr-floxed ($Lepr^{flox/flox}$) mice (obtained from Dr. Streamson. Chua, Albert Einstein College of Medicine), in which exon 17, a critical exon involved in Lepr signaling, is floxed[32], were crossed with a dopamine transporter (DAT, Slc6a3) promoter-driven Cre transgenic mouse line (DAT-Cre)[33] (Figure 1A). Cre is expressed in virtually all midbrain dopamine neurons in this line of DAT-Cre transgenic mice[33]. The $Lepr^{flox/+}$, DAT-Cre offspring were back-crossed with $Lepr^{flox/flox}$ to generate conditional knockout mice, i.e. $Lepr^{flox/flox}$, DAT-Cre ($Lepr^{DAT\text{-}Cre}$) and $Lepr^{flox/flox}$ littermates. DAT expression is restricted to dopamine neurons, and it is highly expressed in the ventral midbrain[34]. The mice were maintained by crossing $Lepr^{DAT\text{-}Cre}$ with $Lepr^{flox/flox}$ mice. Animals from generations F5–6 were used for the experiments in this study.

**X-gal staining—**To evaluate the specificity of DAT-Cre recombinase activity in dopamine neurons, DAT-Cre mice were mated with Rosa-26 reporter mice carrying the Gt(Rosa)26Sortm1Sor allele, in which lacZ expression is driven by the ROSA26 promoter[35]. Double-transgenic mice expressing the Rosa-26 reporter allele and the DAT-Cre allele were identified using PCR-based genotyping. Mice that were positive for both transgenes were transcardially fixed with 4% paraformaldehyde (PFA). The brains were removed, cryoprotected in 30% sucrose, and sectioned at 40 μm. X-gal staining was processed with free-floating tissue sections by incubating in X-gal staining solution (0.1% X-gal, 5 mM K3Fe(CN)6, 5 mM K4Fe(CN)6, 2 mM MgCl2 in PB, pH = 7.4) for 4 h at 37°C. The staining was examined underneath a light microscope.

**RNA extraction and RT-PCR—**Tissue micropunches of the VTA and the entire hypothalamus of $Lepr^{flox/flox}$ mice and $Lepr^{DAT\text{-}Cre}$ mice were homogenized, and total RNA was extracted. SuperScript™ first-strand synthesis system (Invitrogen) was used to generate cDNA using the $oligo(dT)_{25}$ as the template primer. The reaction mixture consisted of 1 μg of total RNA, 500 ng $oligo(dT)_{25}$, 2 μl of 10× First-Strand buffer, 10 mM DTT, 40 units of RNaseOUT™, and 50 units of SuperScript™ II reverse transcriptase. After incubation at 42°C for 50 minutes, the reaction was inactivated by heating at 70°C for 15 minutes. The resulting cDNA was used for PCR amplification of Lepr exon 17 or β-actin with Accuprime *pfx* Supermix (Invitrogen). The conditions for PCR were 94°C for 5 min, followed by 31 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min followed by a final incubation at 72°C for 10 minutes. The primer sequences used to amplify each product are as follows: Lepr exon 17, forward: 5'-GGGACGATGTTCCAAACCCCA-3' and reverse: 5'-AGGCTCCAGAAGAAGAGGACC-3'; β-actin, forward -AGCCATGTACGTAGCCATCC and reverse - TGTGGTGGTGAAGCTGTAGC. The PCR products were analyzed on a 1% agarose gel stained with ethidium bromide.

Real-time PCR was performed on a Realplex2 Mastercycler (Eppendorf). The Ct values for each duplicate were averaged and used for quantification. The amount of mRNA for exon17 for each sample was normalized to β-actin mRNA using the following formula: $2^{(CT\text{exon } 17 - CT\beta\text{-actin})}$.

**Immunohistochemistry—**To confirm the expression of Cre recombinase in dopamine neurons, $Lepr^{DAT\text{-}Cre}$ mice were perfused with 4% PFA. The brains were removed, post-fixed overnight, and then cryoprotected in 30% sucrose and cut into 40-μm coronal sections. Double immunohistochemistry was performed to detect Cre immunoreactivity in neurons positive for tyrosine hydroxylase (TH), a marker for dopamine neurons. Briefly, sections were rinsed three times in PBS, and incubated in blocking buffer (1% BSA, 3% goat serum, 0.3% triton X-100 in PBS) for 1 h. The sections were then incubated with rabbit-anti-Cre antibody (1:500; EMD Biosciences, Madison, WI) and mouse anti-TH antibody (1:1000, Pel-Freez Biologicals, Rogers, AR). After washing in PBS buffer, sections were incubated for 4 h with fluorescent secondary antibodies: Alexa Fluor 488 goat anti-rabbit IgG (1:400, Molecular Probes, Eugene, Oregon) to reveal immunoreactivity for Cre and Alexa Fluor 546 goat anti-mouse IgG (1:400) to reveal immunoreactivity for TH. Finally, the sections were washed in PBS, mounted onto poly-lysine coated glass slides, and coversliped with fluorescence mounting medium.

To confirm the loss of functional Lepr in dopamine neurons, $Lepr^{DAT\text{-}Cre}$ mice and $Lepr^{flox/flox}$ control mice were food deprived overnight received injections with recombinant mouse leptin (5 mg/kg, i.p., R&D systems, Minneapolis, MN). Two hours after leptin injection, mice were transcardially perfused and processed as described above. The brain sections were incubated with rabbit anti-phosphorylated STAT3 (tyr705) (1:3000, Cell Signal Technology) antibody and mouse anti-TH antibody, for 48 h at 4°C, followed by incubation with Alexa Fluor 488-conjugated goat anti-mouse to reveal immunoreactivity of TH and HRP-conjugated goat anti-rabbit antibodies (1:1000, Progema, Madison, WI). The sections were then incubated in the TSA Plus Cy3 reagent (Perkin-Elmer) for visualization of phosphorylated STAT3. The colocalization of phosphorylated STAT3 with TH was visualized using an Olympus FV1000 confocal microscope. The numbers of the phosphorylated-STAT3 labeled cells were counted bilaterally in the VTA from 3 animals per group.

## Behavioral procedures

**Motor and sensory performance—**On the testing day, the mice were transferred to the procedure room, separated into individual clean cages containing a mixture of new bedding and bedding from the original cage and habituated for 2 h. For the assessment of locomotor activity, the cage lid of the mouse cage was replaced by a clear Plexiglass cover, and the cage was placed in the center of a box with an open top. A CCD camera was mounted above the open box for recording locomotor activity of mice for 30 min. The distance travelled every 2-min interval and the total distance travelled in 30 min were measured by using the Noldus EthoVision 3.0 system (Noldus Information Technology Inc., Leesburg, VA). To evaluate motor coordination, mice were tested on the accelerating rotarod (Ugo Basile Biological Research Apparatus Company, PA). Mice were placed on the rotarod apparatus and the rotarod was programmed to accelerate from 0 to 45 rpm over 5 min. The latency to fall from the rod was recorded. Each mouse was tested four times a day for two consecutive days with a 15–30 min inter-trial interval, for a total of 8 trials. The visual ability was measured using the visual cliff test by evaluating their avoidance of a drop-off at the edge of a horizontal surface[36]. A box with an open side and a black floor was placed adjacent to a

vertical drop of 0.7 m. A sheet of clear Plexiglas covered the drop off and provided a solid horizontal surface in spite of the visual appearance of a cliff. The mouse was placed at the edge of the black floor and the direction in which the mouse stepped was recorded. Normal mice step toward the black surface more often than the clear surface whereas mice with impaired vision will step toward the black and clear side with equal frequency. Each mouse was subjected 10 consecutive trials. The olfactory function was examined using a buried food pellet test[37]. Mice were food deprived for 24 h prior to the test. On the test day, a single mouse was placed in a clean cage to recover a food pellet that was buried 0.5 cm below the surface of a 3 cm deep layer of mouse bedding material. The location of the pellet was changed for different animals at random and the latency to find the pellet was recorded. This was defined as the time between placement of the mouse in the cage and grasping the food pellet with its forepaws or teeth.

**Anxiety-related behavioral tests—**A battery of behavioral tests was used to evaluate anxiety-like behaviors. The elevated plus maze is a validated and widely used test of anxiety in rodents[38]. The plus maze consisted of four arms (30-cm long and 5-cm wide) arranged in the shape of a "plus" sign and elevated to a height of 70 cm from the floor. Two arms had no side or end walls (open arms), and the other two arms had side walls and end walls (12-cm high) but were open on top (closed arms). The arms intersected at a central platform (5×5 cm) that allowed access to all of the arms. Mice were placed in the central square facing the corner between a closed arm and an open arm, and allowed to explore the elevated plus-maze for 5 min. The time spent on the open and closed arms and the numbers of entries made into each arm were measured. Entry was defined as all four paws entering one arm. The degree of anxiety was assessed by calculating the percentage of open arm entries (entries into the open arms/total entries into all arms) and percentage of open arm time (time spent in the open arms/total time spent in all arms). The light dark test procedure is based on a natural conflict of a mouse between the innate aversion to brightly illuminated areas and the exploration of a novel environment[39]. The apparatus consists of two compartments (27 × 27 × 30 cm for the light compartment and 18 × 27 × 30 cm for the dark compartment) divided by a wall with a door between the two compartments. The light compartment was brightly illuminated with light intensity of 900 lux; and the dark compartment was black-walled and covered at the top with black Plexiglas. Mice were placed individually in the center of the dark compartment facing away from the opening. The latency for the mice to move to the light side and time spent in the light compartment were recorded for 5 min. Entry to the light compartment was defined as all four paws entering the light side of the box. The social interaction test was carried out with a modification of the method used by File and Hyde[40]. The test apparatus consisted of a white box (40 × 40 × 40 cm) with an open top. The illumination in the test arena was adjusted to 250 lux. Animals were not habituated to the test box prior to testing. Mice were tested for social interaction with an unknown test partner from a different cage but with the same genotype and approximately the same body weight. Two mice were placed simultaneously in the opposite corners of the arena. Their social activity was recorded for 10 min. The active social behaviors were scored including nosing, following, and non-aggressive physical contacts (body sniffing, anogenital sniffing, body crossing). The novelty suppressed feeding test assesses stress-induced anxiety by measuring the latency of an animal to approach and eat a familiar food in a novel

environment[41]. Mice were food deprived for 24 h prior to the test. At the time of testing 1 food pellet was placed on a white filter paper located in the middle of the test arena (60 × 60 × 40 cm) and covered with 2 cm of fresh bedding. Each mouse was placed in one corner and allowed to explore for a maximum of 10 min. The latency to approach the pellet was recorded. Immediately after an eating event, the mouse was transferred to its home cage and allowed to free-feed for 5 minutes. The amount of food consumption was recorded.

**Depression-related tests—**The sucrose preference test has been used as a procedure to measure hedonic response to natural reward in mice[42]. For the basal sucrose preference, mice were habituated to drinking from two bottles of water for one week prior to testing. To measure the preference for sucrose, the animals were singly housed and provided with a free choice of water or sucrose solution (0.2% or 1%, w/v) for 4 consecutive days. Water and sucrose intake was measured daily, and the position of the two bottles was switched every day. Sucrose preference was calculated as a percentage of consumed sucrose solution of the total amount of liquid that the mice drank. For the measurement of sucrose preference in response to chronic unpredictable stress, animals were singly housed and subjected to a variety of stressors applied randomly and at varying times of the day for 14 consecutive days as described previously[29]. The stressors included 30 min restraint, 10 min tail pinch, 1h shaking, 24 h constant light, 24h wet bedding/45° cage tilt, electric footshock, and 2 h social stress. Control non-stressed mice were group housed and handled daily in the housing room. Two bottles of water were placed in each animal cage 2 days prior to and during the chronic unpredictable stress procedure. After 14 days of chronic unpredictable stress, the animals were provided with a free choice between plain water and 1% sucrose. Water and sucrose intake was measured for 2 days and the position of the two bottles was switched every 24 h.

The tail suspension and forced swim tests are widely used for screening antidepressant properties of drugs[43, 44]. In both tests, animals first struggle vigorously in an attempt to escape. However, over time animals become immobile, which is believed to reflect a state of “behavioral despair”. For the tail suspension test, the apparatus was constructed of a wooden box (30×30×30 cm) with an open front. A horizontal bar was placed 1 cm from the top and a vertical 9 cm bar hanging down in the center. Mice were individually suspended by the tail to the vertical bar with adhesive tape affixed 2 cm from the tip of the tail. The animal’s behavior was recorded for 6 min. The immobility and escape oriented behaviors scored by a trained observer who was blind to the treatments. For the forced swim test, mice were placed in a clear Plexiglas cylinder (25 cm high; 10 cm in diameter) filled to a depth of 15 cm with 24°C water. Animals’ behavior in a 6-min test session was recorded. The first 2 min were designated as a habituation period, and the duration of immobility was measured during last 4 min using Noldus EthoVision 3.0 system (Noldus Information Technology Inc., Leesburg, VA).

Adult male mice (8–12 weeks) were used for the anxiety- and depression-related behavioral tests. All behavioral tests were performed in the light cycle, and were scored by experimenters who were blind to the treatments or genotypes. New animals were used for the elevated plus-maze, light dark box, social interaction, novelty suppressed feeding, sucrose preference, tail suspension test and forced swim test. The animals used for

locomotor activity were re-tested for rotarod performance. The mice that were tested for sucrose preference under the basal condition were used for visual cliff and olfactory tests.

## Electrophysiology

Mice were anesthetized with 4% chloral hydrate (400 mg/kg, i.p.) and placed in a stereotaxic apparatus. It has been reported that, under chloral hydrate anesthesia, dopamine neuron activity states more closely resemble that observed in freely moving animals[45]. Anesthesia was maintained by supplemental administration of chloral hydrate as required to maintain suppression of limb compression withdrawal reflex. A core body temperature of 37°C was sustained by a thermostatically controlled heating pad. An incision was made in the midline and a burr hole drilled above the VTA. Recording electrodes were pulled from borosilicate glass capillaries and filled with 2M NaCl containing 2% Pontamine sky blue dye (impedance 6–14 MΩ). Electrodes were lowered into the VTA (Coordinates: 3.2 mm posterior to the bregma, 0.4 mm lateral to the midline, and -5.0 mm ventral to the brain surface) using a hydraulic microdrive and 6–12 vertical passes were made throughout the VTA, separated by 100 μm. Spontaneously active dopamine neurons were identified with open filter settings (30 Hz – 30 kHz) using established electrophysiological criteria[46, 47]. Once isolated, two distinct activity states were measured over a period of 3 min: i) basal firing rate (Hz), and (ii) the proportion of action potentials occurring in bursts (defined as the occurrence of two spikes with an interspike interval of < 80 ms, and the termination of the burst defined as the occurrence of an interspike interval of > 160 ms[48]. At the cessation of the experiments, mice were decapitated and their brains processed for histological verification of electrode tracks.

## Intra-central amygdala microinjection

Mice were anesthetized as described above and implanted unilaterally or bilaterally with a guide cannula (26-gauge; C315GS; Plastics One, Roanoke, VA) positioned 2 mm above the central amygdala (Coordinates: −0.94 mm posterior to bregma, ±2.8 mm lateral to the midline, and −2.8 mm ventral to bregma). A stainless steel dummy cannula was used to seal the guide cannula after surgery. Following cannula implantation, animals were housed individually and allowed to recover for 7 d before performing microinjection. During this recovery period, animals were handled daily to minimize stress caused by the microinjection procedure. All microinjections were performed on conscious, unrestrained, freely moving mice in their home cage. On experimental day, a 33-gauge stainless steel injector connected to a 5-μl syringe was inserted into the guide cannula and extended 2 mm beyond the tip. The D1 dopamine antagonist SCH23390 (Sigma, St. Louis, MO) was dissolved in isotonic sterile saline. SCH23390 (0.3 μg per mouse) or vehicle (saline) was infused in a volume of 0.2 μl per side over 2 min. An additional minute was allowed for diffusion and prevention of backflow through the needle track before the injector was withdrawn. Animals were tested on the elevated plus-maze 30 min after microinjections. After completion of behavioral testing, mice were injected with 0.2 μl of India ink via an injector under anesthesia and sacrificed by decapitation. The fresh frozen brains were sectioned at 20 μm and stained with toluidine blue to verify the placement of the injection needle. Animals with misplacement of cannula were excluded from data analysis.

### Western blot analysis

Mice were decapitated rapidly, and tissue micropunches from the central amygdala, nucleus accumbens, striatum and VTA were homogenized in lysis buffer (50 mM Hepes, pH 7.6, 1% Triton X-100, 150 mM NaCl, 20 mM sodium pyrophosphate, 20 mM β-glycerophosphate, 10 mM NaF) containing a mixture of phosphatase inhibitors (leupeptin, aprotinin, sodium orthovanadate, phenylmethylsulfonyl fluoride, Ser/Thr phosphatase inhibitor mixture, Tyr phosphatase inhibitor mixture). A total amount of 40 μg protein was separated on an SDS-PAGE gel, transferred to a nitrocellulose membrane, and subsequently incubated with rat anti-DAT (1:3000, Chemicon) and mouse anti-β-tubulin antibodies (1: 1000, Chemicon) diluted in a solution of 1% bovine serum albumin and 0.1% Tween 20 in Tris-buffered saline overnight at 4 °C. Next, the membrane was washed and incubated in secondary antibody conjugated to horseradish peroxidase in a blocking solution for 1 h. Immunoblotting results were visualized using an electrogenerated chemiluminescence reaction and exposed to X-ray film.

### Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). Statistical analyses were performed by using one-factor ANOVA with repeated measures on food intake, body weight gain, sucrose preference and motor functions, two-way ANOVA on chronic unpredictable stress-induced anhedonia, and behavioral effects of SCH23390, followed by a post hoc Bonferroni/Dunn or Tukey/Kramer (for unequal n) test. The proportions of high burst-firing cells and low burst-firing neurons were analyzed using Chi-squared test. The rest of experimental results were analyzed using Student *t*-test.

## Results

### Generation of mice lacking Lepr in dopamine neurons

The specificity of DAT-Cre-mediated recombination was confirmed by crossing DAT-Cre mice with Rosa26 reporter mice. X-gal staining indicated that the Cre recombinase activity was restricted to dopamine neuron regions including the VTA and SNc (Figure S1A). Furthermore, double-labeling immunohistochemistry was performed on brain sections of $Lepr^{DAT\text{-}Cre}$ mice and confirmed that Cre immunoreactivity was exclusively localized in neurons positive for the dopamine neuron marker TH (Figure S1B). The effectiveness of deletion of exon 17 was confirmed by RT-PCR and real-time quantitative PCR analyses, which detected a significant reduction of exon 17 of Lepr in the VTA ($t_{(6)} = 2.82$, $p < 0.05$) (Figure 1B,C). In contrast, the hypothalamus, another brain region containing dopamine neurons[49], showed no difference in the levels of Lepr exon 17 mRNA (Figure 1B,C), suggesting that a Cre-mediated recombination of the Lepr-flox allele occur specifically in the midbrain. This is consistent with the report that dopamine neurons that express Lepr are restricted to the midbrain[7]. To validate that the deletion of exon 17 leads to the functional loss of leptin receptors in dopamine neurons, we assessed STAT3 phosphorylation, a functional readout for leptin receptor activation, in $Lepr^{DAT\text{-}Cre}$ mice. Following intraperitoneal (i.p.) injection of leptin (5 mg/kg), phosphorylated STAT3 was observed in VTA dopamine neurons in $Lepr^{flox/flox}$ control mice. In $Lepr^{flox/flox}$ control mice, the majority of the cells positive for phosphorylated STAT3 were also stained for TH

immunoreactivity. However, none of the scattered phosphorylated STAT3 cells in $Lepr^{DAT\text{-}Cre}$ mice were observed to colocalize with TH (Figure 1D), supporting complete ablation of the functional Lepr in dopamine neurons.

## $Lepr^{DAT\text{-}Cre}$ mice display normal body weight, food intake and hedonic responses

Given the role of the mesolimbic dopamine system in food intake and reward, we assessed the effects of inactivation of Lepr on dopamine neurons on appetite, weight regulation and hedonic responses. Body weight was monitored in mice from 3 to 12 weeks of age. There was no significant difference between $Lepr^{DAT\text{-}Cre}$ mice and $Lepr^{flox/flox}$ littermate controls ($F_{(1, 231)} = 0.004$, $p > 0.5$) (Figure 2A). At 13 weeks of age, the intake of standard chow was measured for 7 days. Daily food intake over the 7-day testing period showed no significant effect of genotype ($F_{(1, 102)} = 0.14$, $p > 0.5$ for daily food intake; $t_{(17)} = 0.39$; $p > 0.5$ for average daily food intake) (Figure 2B). Subsequently, $Lepr^{DAT\text{-}Cre}$ mice and $Lepr^{flox/flox}$ mice were tested for the response to a palatable, high fat diet (45% calories from fat) for 7 days. The two genotype groups showed similar daily intake of high fat diet over the 7-day testing period ($F_{(1, 90)} = 1.60$, $p < 0.1$ for daily food intake; $t_{(15)} = 0.75$, $p > 0.1$ for average daily food intake) (Figure 2C). Body weight was measured before and 3 or 7 days after the high fat diet switch. $Lepr^{DAT\text{-}Cre}$ mice and $Lepr^{flox/flox}$ littermate control mice showed similar body weight gain during the high fat diet feeding ($F_{(1,28)} = 0.34$, $p > 0.5$).

Next, we examined the intake and preference for sucrose, a well established natural reward that involves activation of dopamine neurons[50–53]. A free choice between plain water and 0.2% sucrose was first presented to $Lepr^{DAT\text{-}Cre}$ mice and $Lepr^{flox/flox}$ littermate control mice. Then, a higher concentration (1%) of sucrose solution was given to the same mice. The statistical analysis revealed a significant effect of sucrose concentration ($F_{(1,15)} = 21.20$, $p < 0.001$) but no main effect of genotype ($F_{(1,15)} = 0.015$, $p > 0.5$). The interaction between genotype and sucrose concentration was not significant ($F_{(1,15)} = 0.89$, $p > 0.3$) (Figure 2D). In addition, $Lepr^{DAT\text{-}Cre}$ mice and $Lepr^{flox/flox}$ littermate controls showed no significant difference in daily sucrose intake for either 0.2% sucrose solution ($Lepr^{flox/flox}$: 2.13 ± 0.22 ml; $Lepr^{DAT\text{-}Cre}$: 2.40 ± 0.42 ml) or for 1% sucrose solution ($Lepr^{flox/flox}$:3.33 ± 0.48 ml; $Lepr^{DAT\text{-}Cre}$: 3.83 ± 0.39 ml). Taken together, these data suggest that leptin receptor signaling in dopamine neurons may not be required for appetitive behavior and the reward processes manifested by palatable high fat food or sucrose solution.

To rule out the possible confounding effect of the Cre transgene in $Lepr^{DAT\text{-}Cre}$ mice, body weight gain, food intake, including regular chow and high fat diets, and sucrose preference were evaluated in DAT-Cre transgenic mice in comparison with wild-type littermate controls. None of these measures showed a significant difference between Cre mice and wild-type littermate controls (Figure S2).

## $Lepr^{DAT\text{-}Cre}$ mice display normal motor and sensory performance

Given that most behavioral evaluations are dependent on locomotor activity, we examined locomotor activity in the late light cycle when most behavioral tests were performed. $Lepr^{DAT\text{-}Cre}$ mice and $Lepr^{flox/flox}$ littermate controls exhibited similar locomotor activity during the 30-min test [($F_{(1, 252)} = 0.046$, $p > 0.5$) for the time course of distance traveled

every 2 min; $t_{(18)} = 1.44$; $p > 0.1$ for the total distance traveled] (Figure 3A). In addition, motor coordination was evaluated using a rotarod test. The ability to perform or learn an accelerating rotarod task was unaltered in $Lepr^{DAT\text{-}Cre}$ mice (Figure S3A). These results suggest that a loss of leptin signaling in dopamine neurons does not affect motor functions. In addition, no gross impairments in their visual and olfactory capacities in $Lepr^{DAT\text{-}Cre}$ mice were observed at the age when behavioral tests were performed (Figure S3B, C).

## $Lepr^{DAT\text{-}Cre}$ mice exhibit an anxiogenic phenotype

To evaluate the effects of Lepr deletion in dopamine neurons on anxiety-related behaviors, mice were examined in a series of behavioral tests. In the elevated plus-maze test, $Lepr^{DAT\text{-}Cre}$ mice displayed a significantly lower percentage of open arm entries ($t_{(18)} = 3.35$; $p < 0.01$) and time spent in the open arms ($t_{(18)} = 3.57$; $p < 0.01$) without affecting total arm entries ($Lepr^{flox/flox}$: 13.60 ± 1.431; $Lepr^{DAT\text{-}Cre}$: 11.60 ± 1.108; $t_{(18)} = 1.11$; $p > 0.2$), suggesting increased anxiety levels in $Lepr^{DAT\text{-}Cre}$ mice (Figure 3B). In the light dark box, $Lepr^{DAT\text{-}Cre}$ mice explored the light compartment to a significantly lesser extent than $Lepr^{flox/flox}$ littermate controls, as measured by increased latency to enter the lit side ($t_{(22)} = 3.19$; $p < 0.01$) and decreased time spent in the lit side ($t_{(22)} = 3.12$; $p < 0.01$) (Figure 3C). In the social interaction test, two mice with the same genotype that had similar body weight and were unfamiliar to each other were simultaneously placed in a novel test arena[28]. $Lepr^{DAT\text{-}Cre}$ mice spent significantly less time in active social interaction compared to $Lepr^{flox/flox}$ littermate controls ($t_{(19)} = 2.21$; $p < 0.05$) (Figure 3D). The novelty suppressed feeding test required food deprivation for 24 h followed by testing the animal's latency to approach food pellets that were placed in the center of a novel, open and brightly lit arena. $Lepr^{DAT\text{-}Cre}$ mice exhibited significantly longer latencies to begin feeding when compared with $Lepr^{flox/flox}$ littermate controls ($t_{(22)} = 2.28$; $p < 0.05$), while feeding activity in the home cage was similar (Figure 3E).

## Depression-related behaviors are unaltered in $Lepr^{DAT\text{-}Cre}$ mice

Sucrose preference has been widely used for assessing 'anhedonia', a symptom of human depression, which can be induced by chronic stress in rodents[29, 54–57]. To determine if deletion of Lepr signaling in dopamine neurons influences the sensitivity to chronic stress-induced anhedonia, mice were exposed to a 2-week chronic unpredictable stress procedure. Sucrose preference in $Lepr^{DAT\text{-}Cre}$ mice and $Lepr^{flox/flox}$ littermate controls was tested using 1% sucrose solution. The statistical analysis indicated a significant effect of chronic unpredictable stress ($F_{(1, 33)} = 17.34$; $p < 0.001$), but not genotype ($F_{(1, 33)} = 0.58$; $p > 0.1$). There was no significant effect of genotype × chronic unpredictable stress ($F_{(1, 33)} = 0.28$; $p > 0.1$) (Figure 4A). In the tail suspension test and forced swim test, immobility time, an index of 'behavioral despair', was unaltered in $Lepr^{DAT\text{-}Cre}$ mice in comparison with $Lepr^{flox/flox}$ littermate controls (Tail suspension: $t_{(17)} = 0.81$; $p > 0.1$; Forced swim: $t_{(15)} = 0.56$; $p > 0.5$) (Figure 4B, C). Taken together, these behavioral data indicate that a loss of Lepr in dopamine neurons has no effect on depression-related behaviors.

To rule out the possible confounding effects of the Cre transgene on emotional behaviors, we tested DAT-Cre mice in comparison with wild-type littermate controls. The DAT-Cre transgenic mice and wild-type mice were indistinguishable in the behavioral tasks as

described above (Figure S4). Furthermore, to rule out the possibility that a change in DAT protein might contribute to the behavioral outcome in $Lepr^{DAT\text{-}Cre}$ mice, we measured the levels of DAT in different terminal regions of dopamine neurons, including the central amygdala, nucleus accumbens, striatum, and VTA of $Lepr^{DAT\text{-}Cre}$ mice at 9 weeks of age. DAT protein levels were similar in $Lepr^{DAT\text{-}Cre}$ mice and $Lepr^{flox/flox}$ littermate control mice (Figure S5).

## $Lepr^{DAT\text{-}Cre}$ mice show increased burst firing activity of dopamine neurons in the VTA

To understand the mechanisms underlying the behavioral effects of dopamine neuron-specific Lepr deletion, we investigated the electrophysiological profiles of dopamine neurons in the VTA. Single-unit extracellular recordings were performed in chloral hydrate anesthetized $Lepr^{DAT\text{-}Cre}$ mice and $Lepr^{flox/flox}$ littermate controls. Spontaneously active dopamine neurons were identified with open filter settings (low pass: 30 Hz, high pass: 30 kHz) using previously established electrophysiological criteria, including an action potential duration > 2 ms ($Lepr^{flox/flox}$: 2.9 ± 0.1 ms; $Lepr^{DAT\text{-}Cre}$: 3.2 ± 0.3 ms)[46]. $Lepr^{flox/flox}$ mice displayed an average firing rate of 3.99 ± 0.30 Hz with 30.4 ± 3.7 % of action potentials occurring in bursts (Figure 5A, B, E, F), consistent with previous findings in untreated animals[58, 59]. $Lepr^{DAT\text{-}Cre}$ mice showed no difference in the average firing rate (4.95 ± 0.51 Hz) (Figure 5E), however, these mice exhibited a significantly greater burst firing (48.3 ± 5.2 %; $p < 0.05$. Figure 5C, D, F). Burst-firing frequency distribution of cells recorded showed a significant increase in the number of high burst-firing cells with a concomitant decrease in low burst-firing neurons in $Lepr^{DAT\text{-}Cre}$ mice ($p < 0.05$; Figure 5G, H). The electrode tract throughout the VTA was confirmed with histological staining (Figure 5I, J), targeting the caudal aspect of the VTA where a high degree of colocalization of Lepr and TH was reported[7]. These observations suggest that a loss of leptin signaling in dopamine neurons results in an augmented dopamine neuronal activity.

## Dopamine transmission via D1 receptors in the central amygdala mediates anxiogenic-like behavior in $Lepr^{DAT\text{-}Cre}$ mice

To test whether dopamine transmission in the central amygdala contributes the anxiogenic phenotype of $Lepr^{DAT\text{-}Cre}$ mice, we examined whether blockade of dopaminergic transmission in the central amygdala would alleviate the anxiogenic-like behavior in the elevated plus maze test. The D1 antagonist SCH23390 or vehicle was infused unilaterally or bilaterally into the central amygdala of freely moving mice. Thirty min after drug injection, mice were tested on the elevated plus maze for 5 min. Similar results were observed in mice that received unilateral or bilateral injection of SCH23390 into the central amygdala, the data were combined. Statistical analysis revealed no significant main effect of genotype ($F_{(1,57)} = 3.56$, $p > 0.05$ for percentage of open arm entries; $F_{(1,57)} = 3.90$, $p > 0.05$ for percentage of open arm time), but showed significant effects of treatment ($F_{(1,57)} = 18.62$, $p < 0.001$ for percentage of open arm entries; $F_{(1,57)} = 14.97$, $p < 0.001$ for percentage of open arm time) and interaction between genotype and treatment ($F_{(1,57)} = 5.47$, $p < 0.05$ for percentage of open arm entries; $F_{(1,57)} = 4.57$, $p < 0.05$ for percentage of open arm time). *Post-hoc* analyses showed that percentage of open arm entries and open arm time in vehicle-treated $Lepr^{DAT\text{-}Cre}$ mice was significantly lower than that of vehicle-treated $Lepr^{flox/flox}$ control mice ($p < 0.01$), and SCH23390 treatment significantly increased the percentage of

open arm entries and open arm time in Lepr$^{DAT-Cre}$ mice ($p < 0.001$) (Figure 6). Together, these results suggest that the anxiogenic effect of ablation of Lepr signaling in dopamine neurons may be mediated by D1 dopamine transmission in the central amygdala.

## Discussion

This study demonstrates that the selective inactivation of Lepr in dopamine neurons in mice results in enhanced anxiogenic-like behaviors and increased excitability of dopamine neurons in the VTA. The anxiogenic-like phenotype of mutant mice is likely mediated by dopamine D1 transmission in the central amygdala as blockade of D1 dopamine receptors in this area attenuates the anxiogenic-like behavior. By contrast, depression-related behavior and feeding behavior are not affected by ablation of Lepr in dopamine neurons. These data suggest that leptin receptor signaling in dopamine neurons plays a critical role in modulating anxiety-related behaviors.

Although previous studies have suggested that the midbrain is one extra-hypothalamic site where leptin regulates normal food intake and hedonic feeding[14, 27], the specific role of Lepr signaling in midbrain dopamine neurons in feeding behavior was not clear. In this study, we showed that Lepr$^{DAT-Cre}$ mice lacking Lepr selectively in dopamine neurons exhibited normal body weight gain and feeding behavior. The intake of standard chow and high palatable foods including high fat diet and sucrose solutions (0.2% and 1%) was unaltered in Lepr$^{DAT-Cre}$ mice, suggesting that leptin receptor signaling in dopamine neurons is not required for homeostatic or hedonic feeding. The difference between our results and the previous report by Hommel et al. using AAV-mediated Lepr knockdown in rats, which targeted both dopamine and non-dopamine neurons in the midbrain[14], implies that Lepr on non-dopamine neurons might account for feeding behavior. However, this difference could also be due to species differences between mice and rats. While we cannot exclude the possibility of developmental compensation that might have occurred in Lepr$^{DAT-Cre}$ mice, the lack of feeding and body weight phenotypes in these mice is in contrast to other lines of Lepr conditional knockout mice, such as those mice with loss of Lepr in POMC neurons, SF1 neurons and Nkx2.1 neurons, that develop obesity despite having neuron-specific Lepr deficiency at early stages of development[8, 10, 60, 61]. Recent studies in leptin-deficient subjects suggest that neural circuits through which leptin regulates hedonic feeding behavior might involve specific limbic cortical and subcortical areas. Using functional magnetic resonance imaging (fMRI), Baicy et al. reported that leptin replacement in adults with congenital leptin deficiency reduced activation of the brain regions involved with hunger (insula, parietal and temporal cortex) and increased activation of regions involved in cognitive inhibition and satiety (prefrontal cortex) in response to food cues[62]. Another fMRI study demonstrated that leptin-deficient adolescents displayed activation of the ventral striatum to images of food, which was attenuated after 1 week of leptin treatment[63]. Whether these brain regions are direct leptin targets and whether they mediate leptin action on hedonic feeding requires further investigation. Moreover, leptin replacement in leptin-deficient patients has been shown to exert a sustained effect on cortical structural organization[64] and promote cognitive development[65]. How these structure and functional changes induced by leptin contribute to rewarding responses to food stimuli remain to be explored.

A robust anxiogenic phenotype was observed in Lepr$^{DAT-Cre}$ mice. This was consistently revealed in multiple behavioral tests including the elevated plus-maze, light-dark box, social interaction and novelty suppressed feeding tests. The anxiogenic behavior is unlikely to be a result of general hypolocomotion, sensory abnormalities or altered feeding activity as motor and sensory abilities as well as food consumption in Lepr$^{DAT-Cre}$ mice were not different from Lepr$^{flox/flox}$ littermate controls. Although anxiety and depression often co-occur[66], depression-related behaviors were not affected in Lepr$^{DAT-Cre}$ mice, as evidenced by the absence of an anhedonic phenotype in the sucrose preference test under both basal and chronic stress conditions. In addition, the Lepr$^{DAT-Cre}$ mice exhibited normal performance in the tail suspension test and forced swim test, two procedures widely used for evaluating "behavioral despair". These findings suggest that the modulation of anxiety- and depression-related behaviors by leptin may be mediated via distinct neural circuits. The data from anxiety- and depression-related behavioral tests in Lepr$^{DAT-Cre}$ mice were unlikely to be confounded by the DAT-Cre transgene because DAT-Cre mice did not significantly differ from wild-type littermate mice in these behavioral tests.

Dopamine neuronal activity in the VTA has been implicated in fear and anxiety-like states. A considerable number of dopamine neurons are excited by aversive stimuli[67], and stressful events are associated with mesolimbic dopamine release[68–72]. On the other hand, electrical stimulation of VTA neurons produces fear and anxiogenic responses, whereas lesions of the VTA or inhibition of VTA dopamine neurons by dopamine D2 agonists have anxiolytic effects[73–76]. Anatomical studies have demonstrated that the localization of Lepr in dopamine neurons is restricted to the midbrain with the majority of Lepr-expressing dopamine neurons distributed in the VTA[7]. To understand the mechanisms underlying the anxiogenic phenotype of mice lacking Lepr in dopamine neurons, we recorded the firing activity of dopamine neurons from the VTA *in vivo* and found that the burst firing of VTA dopamine neurons was increased in Lepr$^{DAT-Cre}$ mice. The distribution of burst-firing frequency indicated that a subset of dopamine neurons (20~30%) in the VTA were affected in the Lepr$^{DAT-Cre}$ mice. VTA dopamine neurons are heterogeneous in cortical and sub-cortical projections and functional properties[77]. Our electrophysiological recordings were not limited to dopamine neurons projecting a specific mesolimbic structure. A very recent study by Leshan et al. demonstrates that Lepr neurons in the VTA densely innervate the central amygdala[15], a target area of VTA dopamine neurons[78–80]. Given that the majority of Lepr neurons in the VTA are dopaminergic, we thus posit that altered VTA dopamine neuronal firing properties in Lepr$^{DAT-Cre}$ mice may lead to changes in dopamine transmission in the central amygdala.

The amygdala is a key component essential for the processing of emotions[81, 82]. This structure consists of a group of anatomically and functionally distinct nuclei[83]. Especially, the central nucleus of the amygdala receive convergent information from other amygdaloid subnuclei and elicit an output that reflects the sum of amygdaloid activity[84]. Mesoamygdaloid dopamine neurons have been considered as a neurologic substrate in anxiety and stress responses[85–87]. Indeed, midbrain dopamine neurons increase firing in response to aversive stimuli[88–90], and dopamine transmission in the amygdala is elevated in response to a variety of stress conditions such as inescapable electrical footshock,

conditioned fear and chronic restraint stress[86, 87, 91, 92]. Both dopamine D1 and D2 receptors are expressed in the central amygdala[93–97]. However, D1 receptors have been consistently reported to modulate anxiety behaviors[98–101]. Blockade of D1 receptors in the central amygdala by local infusion of the D1 selective antagonist SCH23390 attenuated the anxiogenic phenotype of $Lepr^{DAT\text{-}Cre}$ mice in the elevated plus-maze test. The effect of SCH23390 could include a mixture of postsynaptic and presynaptic mechanisms, blocking D1 receptor activity localized at postsynaptic elements of central amygdala neurons as well as at presynaptic nerve terminals. Indeed, in addition to sending prominent efferents to the central amygdala, VTA Lepr neurons were also found to project to the other components of the extended amygdala namely the bed nucleus of stria terminalis (BNST) and the interstitial nucleus of the posterior limb of the anterior commissure (IPAC), where dopamine D1 receptors are expressed[78, 102, 103]. These two areas in turn project to the central amygdala[104–106], thereby possibly providing presynaptic axon terminals equipped with D1 receptors. However, given the anatomical proximity and functional connectivity of these two areas with the amygdala, their direct involvement in dopamine regulation of anxiety behavior cannot be ruled out. Nonetheless, the attenuation of the anxiogenic phenotype by blockade of D1 dopaminergic transmission in the central amygdala of $Lepr^{DAT\text{-}Cre}$ mice, together with the finding of increased burst firing of VTA dopamine neurons, supports that increased VTA dopaminergic input to the amygdala is likely an underlying mechanism of anxiogenesis.

In summary, this study demonstrates that Lepr signaling in midbrain dopamine neurons has important actions on anxiety-related behavior that are distinct from the neuronal targets by which leptin regulates feeding behavior or depression-related behavior. Our results provide evidence that the modulation of dopamine neuronal activity by Lepr signaling may represent a novel mechanism for the genesis of anxiety disorders.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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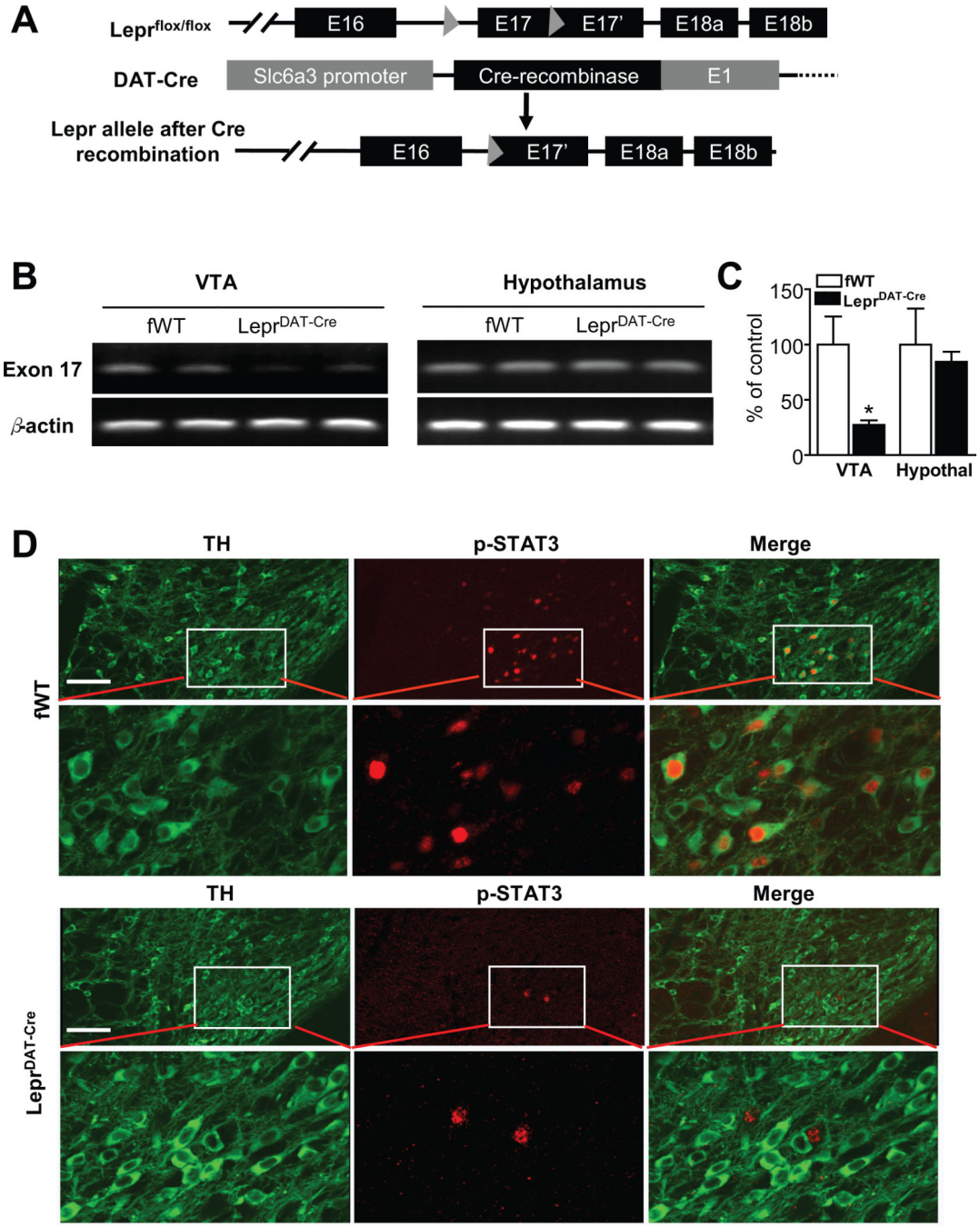


**Figure 1.**

Generation of mice lacking Lepr selectively in dopamine neurons. (A) Schematic diagram depicting the floxed Lepr allele, the Slc6a3 (or DAT) Cre allele, and the Lepr floxed allele after recombination. (B) RT-PCR detection of exon 17 of the leptin receptor in the ventral tegmental area (VTA) versus hypothalamus in $Lepr^{DAT\text{-}Cre}$ mice and $Lepr^{flox/flox}$ littermate control (fWT) mice. (C) Real-time quantitative PCR analysis showing a Cre-mediated deletion of exon 17 of Lepr in the VTA of $Lepr^{DAT\text{-}Cre}$ mice. Values are expressed as a percentage change from fWT control mice. Data are expressed as mean ± SEM. n = 4 per

group. *$p < 0.05$ compared with fWT control mice. (D) Double-labeling immunohistochemistry showing the colocalization of phosphorylated STAT3 in dopamine neurons, positive for tyrosine hydroxylase (TH), in fWT control mice and $Lepr^{DAT\text{-}Cre}$ mice. The loss of Lepr in dopamine neurons eliminates leptin-stimulated phosphorylation of STAT3 in dopamine neurons in $Lepr^{DAT\text{-}Cre}$ mice. Scale bar = 100 μM.

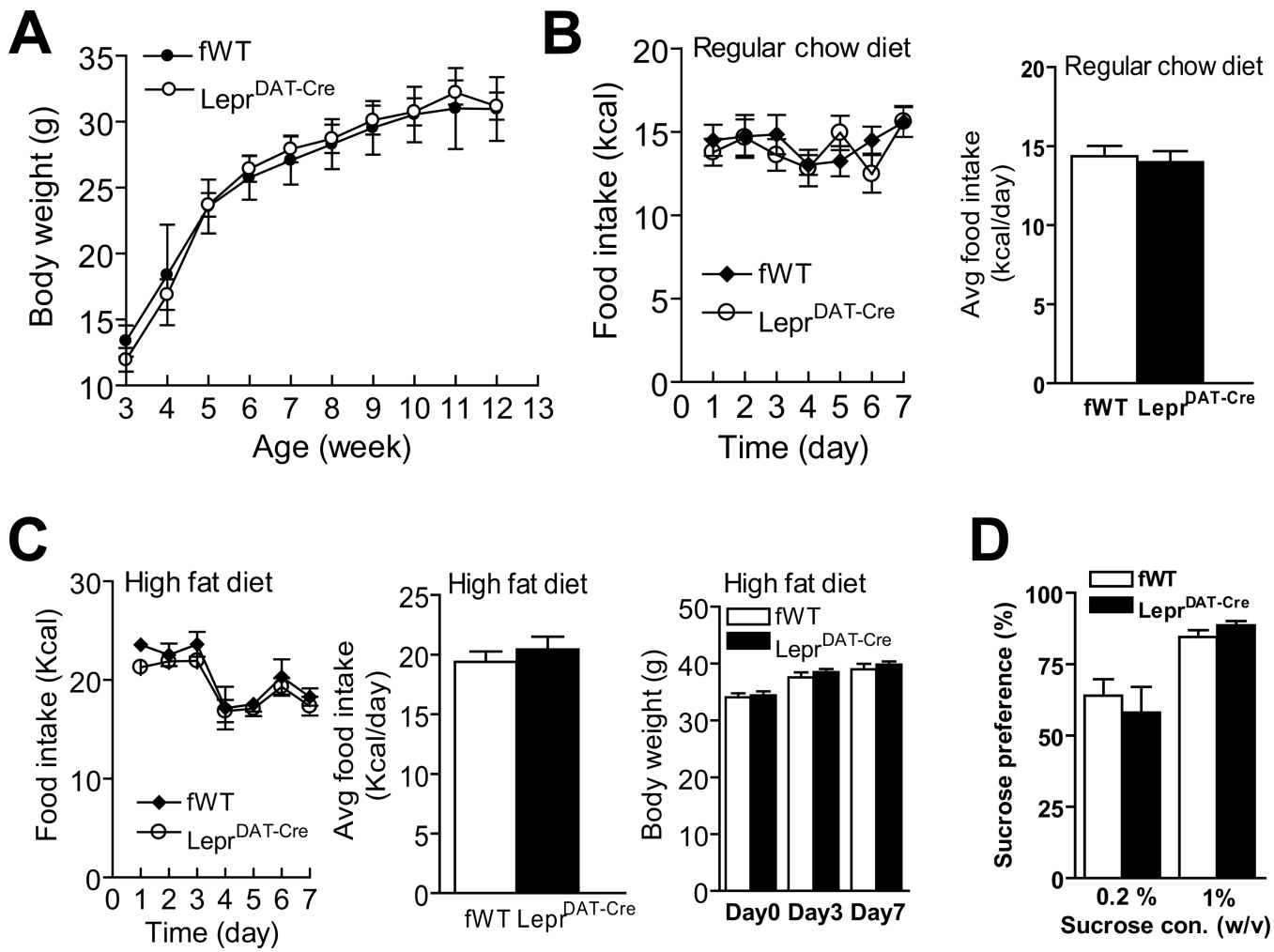


**Figure 2.**

Normal body weight gain, food intake and hedonic responses in Lepr$^{DAT-Cre}$ **mice**. (A) Body weight. (B) Intake of regular chow diet. Left panel: Daily food intake. Right panel: Average food intake over 7 days. n = 9–10 per group. (C) Intake of high fat diet. Left panel: Daily food intake. Middle panel: Average food intake over 7 days. Right panel: Body weight. n = 8–9 per group. (D) Sucrose preference. The preference for sucrose was expressed as a percentage of the volume of sucrose solution intake to the volume of total fluid intake within a 4-day test. n = 8–9 per group. All data are presented as mean ± SEM.

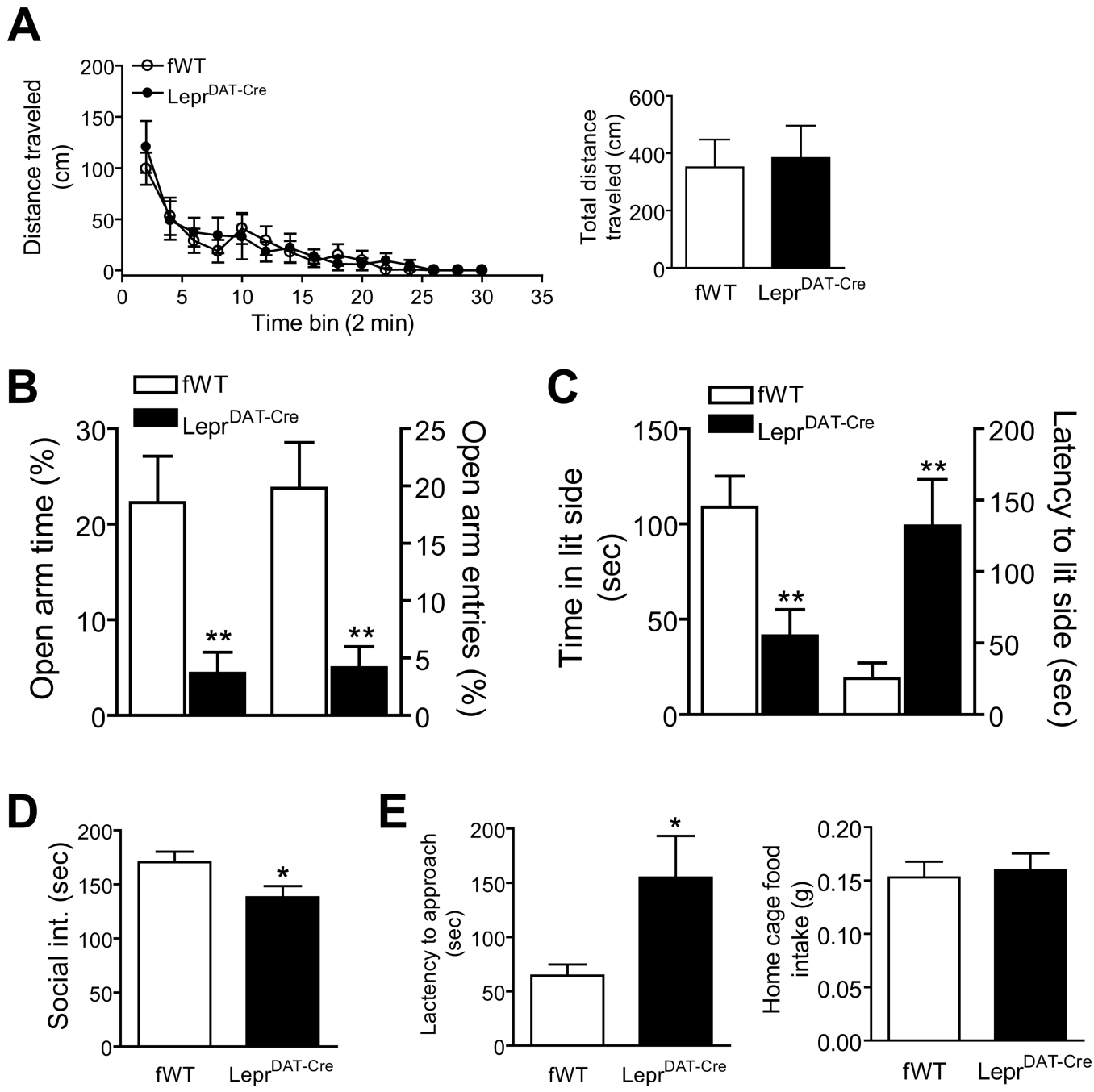


**Figure 3.**

Anxiogenic-like behavioral phenotype of Lepr$^{DAT-Cre}$ mice. (A) Locomotor activity. Left panel: locomotor activity monitored for 30 min and analyzed at 2 min intervals. Right panel: Total distance traveled within 30 min. n = 10 per group. (B) Elevated plus-maze test. Left axis, the percentage of open arm time. Right axis, the percentage of open arm entries. n = 10 per group. (C) Light/dark choice test. Left axis, latency to enter the light side. Right axis, time spent in the light compartment. n = 12 per group. (D) Social interaction test. n = 9–12 pairs per group. (E) Novelty-suppressed feeding test. Left panel, latency to approach food

pallets in the center of an open arena. Right panel, home cage food intake for 5 min immediately following the test. n = 12 per group. All data are expressed as mean ± SEM. *$p < 0.05$; **$p < 0.01$ compared with $Lepr^{flox/flox}$ littermate control (fWT) mice by two-tailed Student's *t* test.

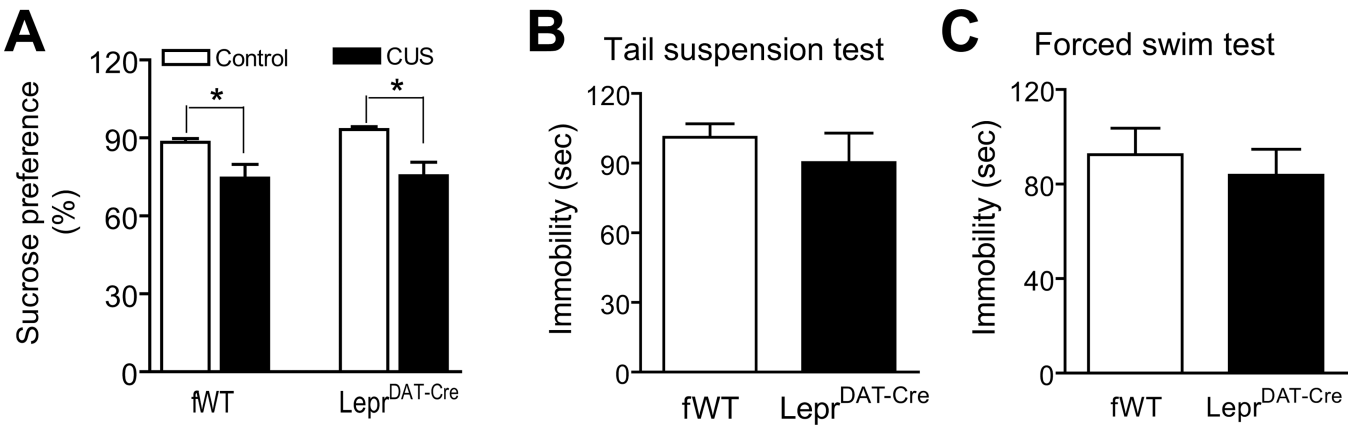


**Figure 4.**

Normal depression-related behavior in Lepr$^{DAT-Cre}$ mice. (A) Sucrose preference following 2 weeks of chronic unpredictable stress (CUS). n = 8–12 per group.*$p < 0.05$ compared with non-stressed groups. (B) Tail suspension test. Immobility time was measured during a 6-min test period. n = 9–10 per group. (C) Forced swim test. Immobility time was measured during the last 4 min of a 6-min test period. n = 8–9 per group. All data are expressed as mean ± SEM.

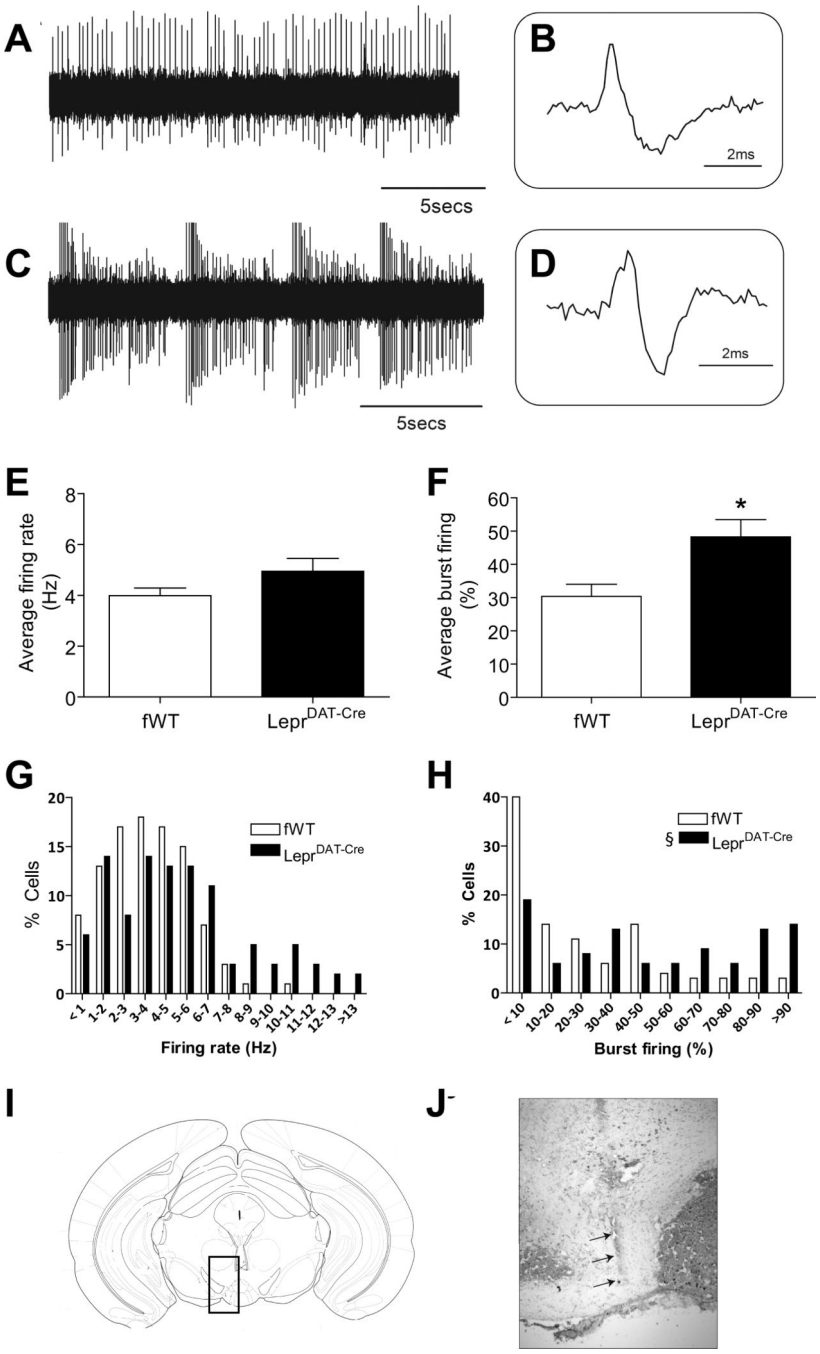


**Figure 5.**

Lepr$^{DAT-Cre}$ mice display enhanced VTA dopamine neuronal activity. Representative voltage traces from identified dopamine neurons demonstrate an irregular, single spike firing pattern of dopamine neurons in the VTA of a Lepr$^{flox/flox}$ mouse (fWT, A & B) and a high burst-firing activity state in a Lepr$^{DAT-Cre}$ mouse (C & D). (E) Average firing rate. (F) Burst firing (G) Firing rate distributions. (H) Burst-firing frequency distributions. Data are expressed as mean ± SEM. n = 7–10 mice, 64–72 neurons per group. (I–J) A representative electrode track throughout the VTA. *$p < 0.05$ compared to Lepr$^{flox/flox}$ littermate controls.

§ representing a significant difference in proportions of high burst-firing cells and low burst-firing neurons, $p < 0.05$.

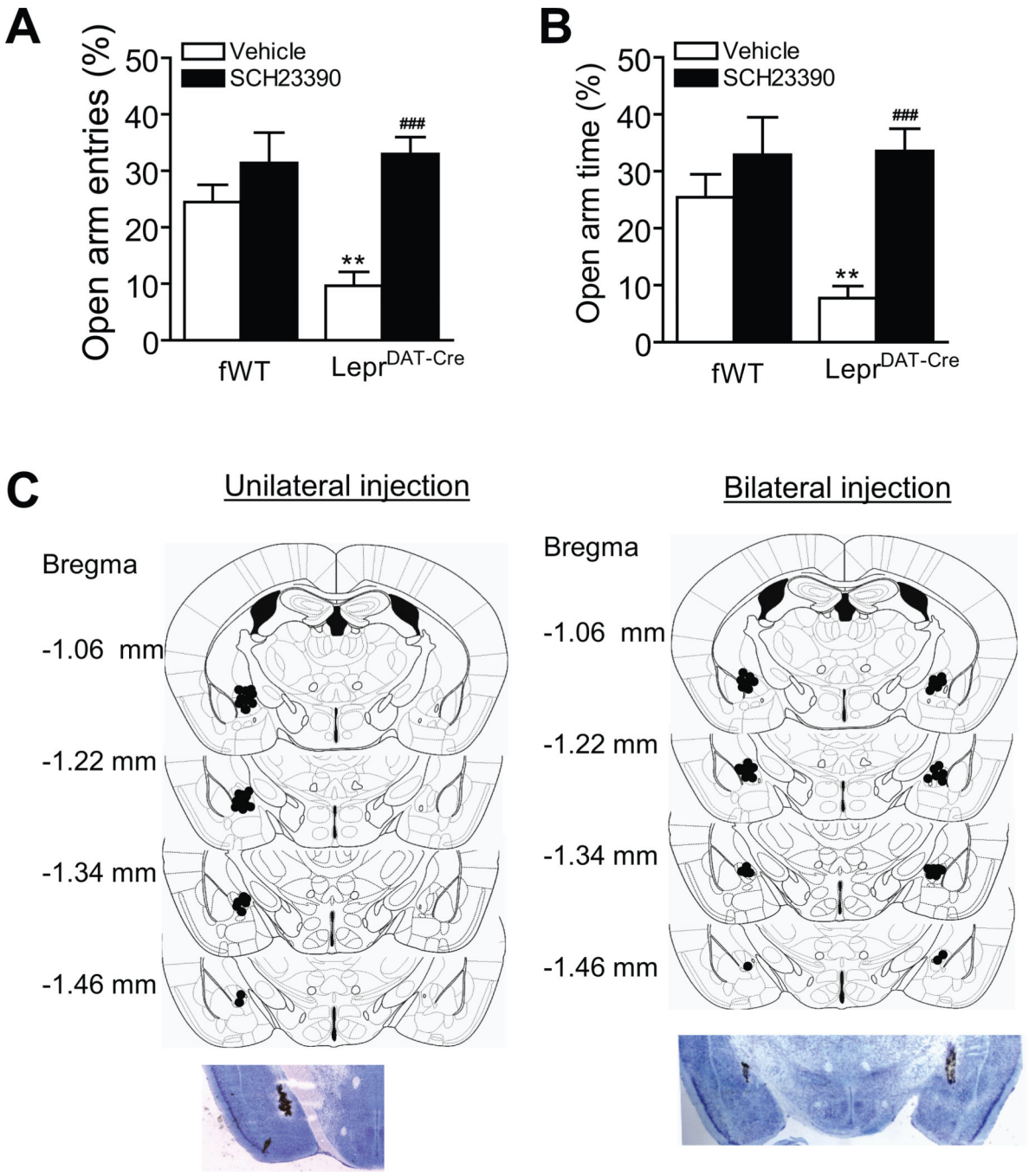


**Figure 6.**

Effect of unilateral and bilateral intra-central amygdala microinjection of the D1 dopamine receptor antagonist SCH23390 on anxiety-like behavior in the elevated plus-maze test. (A) The percentage of open arm entries. (B) The percentage of time spent in the open arms. n = 13–16 mice per group. $^{**}p < 0.01$ compared with the vehicle-treated fWT littermate control group;$^{\#\#}p < 0.01$,$^{\#\#\#}p < 0.001$ compared with the vehicle-treated $Lepr^{DAT\text{-}Cre}$ group. (C) Schematic illustration of unilateral (left panel) and bilateral (right panel) injection sites in

the central amygdala (top) and representative ink injections showing the deposition site within the central amygdala (bottom). All data are expressed as mean ± SEM.