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ORIGINAL ARTICLE

Re-examining the role of ventral tegmental area dopaminergic neurons in motor activity and reinforcement by chemogenetic and optogenetic manipulation in mice

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Abstract

The precise contributions of ventral tegmental area (VTA) dopaminergic (DAergic) neurons to reward-related behaviors are a longstanding hot topic of debate. Whether the activity of VTA DAergic neurons directly modulates rewarding behaviors remains uncertain. In the present study, we investigated the fundamental role of VTA DAergic neurons in reward-related movement and reinforcement by employing dopamine transporter (DAT)-Cre transgenic mice expressing hM3Dq, hM4Di or channelrhodopsin 2 (ChR2) in VTA DAergic neurons through Cre-inducible adeno-associated viral vector transfection. On the one hand, locomotion was tested in an open field to examine motor activity when VTA DAergic neurons were stimulated or inhibited by injection of the hM3Dq or hM4Di ligand clozapine-N-oxide (CNO), respectively. CNO injection to selectively activate or inhibit VTA DAergic neurons significantly increased or decreased locomotor activity, respectively, compared with vehicle injection, indicating that VTA DAergic neuron stimulation is directly involved in the regulation of motor activity. On the other hand, we used the optical intracranial self-stimulation (oICSS) model to investigate the causal link between reinforcement and VTA DAergic neurons. Active poking behavior but not inactive poking behavior was significantly escalated in a frequency- and pulse duration-dependent manner. In addition, microdialysis revealed that the concentration of dopamine (DA) in the nucleus accumbens (NAc) was enhanced by selective optogenetic activation of VTA DAergic neurons. Furthermore, systemic administration of a DA D1 receptor antagonist significantly decreased oICSS reinforcement. Our research profoundly demonstrates a direct regulatory role of VTA DAergic neurons in movement and reinforcement and provides meaningful guidance for the development of novel treatment strategies for neuropsychiatric diseases related to the malfunction of the reward system.

Keywords Ventral tegmental area · Dopamine · Chemogenetic · Optogenetic · Motor activity · Reinforcement

Introduction

All types of addictive drugs directly or indirectly increase the level of the neurotransmitter dopamine (DA), causing a strong sense of euphoria, which indicates that DA is involved in reward and plays an important role in addiction. DA is mainly synthesized by dopaminergic (DAergic) neurons in the ventral tegmental area (VTA); thus, DAergic neurons in the VTA are widely involved in reward-related processes. Previous studies have indicated that DA is critical for modulating locomotion and reinforcement. However, this well-accepted view has been challenged by recent findings in electrophysiological studies supporting the reward prediction error hypothesis (Schultz 1998, 2016) and incentive salience hypothesis (Berridge and Robinson 1998; Berridge and Kringelbach 2015), which propose that the function of DAergic neurons in the VTA is not to mediate reward itself but to convert the neural representations of conditioned stimuli into an attractive and desired incentive, which may be involved in the learning and action selection aspect of reward behavior. Therefore, the precise contributions of VTA DAergic neurons to rewarding behaviors are still unclear.

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Reward-related behavior usually involves an enhancement in motor activity and a positive reinforcement process. In previous studies, acute administration of psychostimulants increased the level of DA, which leads to striking hyperactivity in animals and humans (Garcia-Ruiz et al. 2019; Minogianis et al. 2018; Schrantee et al. 2016). In addition, genetic manipulations that changed the brain DA level altered motor activity (Giros et al. 1996; Xu et al. 1994). In humans, malfunctions of the DA system are associated with several neurological and neuropsychiatric disorders that manifest in dysfunctional motor activity as the presenting symptom (Emilien et al. 1999; Olanow and Tatton 1999). Thus, the modulation of motor activity is believed to be based on DA but not directly on DAergic neurons in the VTA. Recently, Wang's study tested the function of DAergic neurons in motor activity through a chemogenetic approach, determining that specific excitation of DAergic neurons induced hyperactivity (Wang et al. 2013). However, the research neither distinguished the role of DAergic neurons in the VTA and substantia nigra pars compacta (SNc) nor investigated the influence on locomotion induced by the inhibition of DAergic neurons in the VTA. Previous research on reinforcement has shown that systemic administration of addictive drugs (psychostimulants, opioids and alcohol) or intracranial microinjection of nicotine, morphine or alcohol into the VTA induces self-administration behavior, which is associated with upregulation of the DA level in the nucleus accumbens (NAc) (David et al. 2008; Devine and Wise 1994; Ericson et al. 2008; McBride et al. 1999). Furthermore, specific activation of VTA DAergic neurons by optogenetic manipulation leads to self-stimulation behavior in rats (Adamantidis et al. 2011; Kim et al. 2012; Tsai et al. 2009). Nerveless, these studies were designed such that each nose poke, which resulted in the stimulation of DAergic neurons, also generated a simultaneous presentation of a visual cue. Thus, whether the activation of VTA DAergic neurons reinforced the instrumental action directly or by associating with cues acting as a conditioned reinforcement remains blurred.

Therefore, in this study, we specifically addressed the role of VTA DAergic neurons in reward-related motor activity and reinforcement by employing a chemogenetic approach and optogenetic stimulation rather than classical pharmacological approaches. Optogenetic and chemogenetic tools enable temporally precise manipulation of VTA DAergic neurons in a cell-type specific manner. First, we investigated the role of VTA DAergic neurons in motor activity by using designer receptor exclusively activated by designer drugs (DREADD)-induced durable, reversible and selective DAergic manipulation. Second, the transient and reversible modulation offered by optogenetics was applied to induce self-stimulation of VTA DAergic neurons with multiple frequencies and pulse durations to determine the relationship between reinforcement and the activity of DAergic neurons in the VTA. Moreover, we also measured the concentration of DA while VTA DAergic neurons were optogenetically activated. Finally, DA D1 receptor antagonist was systemically injected to intervene the optical intracranial self-stimulation behavior to determine the receptor involved in reinforcement.

Materials and methods

Animals

All experiments were performed with adult dopamine transporter (DAT)-Cre BAC transgenic mice (strain B6.SJL-Slc6a3tm1.1 (cre)Bkmn/J, Jackson Laboratory, Bar Harbor, Maine, USA). Mice weighing 25–30 g were individually housed in temperature- and humidity-controlled rooms on a light-dark cycle (22 °C, 50–60% humidity, 12:12-h light/dark cycle with lights on at 8:00 a.m. and lights off at 8:00 p.m.). The animals received food and water ad libitum, except during the experimental sessions. All animal treatments were performed in strict accordance with the guidelines of the Institutional Review Committee for the Use of Animals.

Drugs

Clozapine nitrogen-oxide (CNO, Sigma-Aldrich LLC.) used in the chemogenetic strategy was dissolved in 2% dimethyl sulfoxide (DMSO) to obtain the indicated doses (1 mg/kg). The DA D1 receptor antagonist SCH23390 (Sigma-Aldrich LLC.) was dissolved in saline to 0.2 mg/kg for intraperitoneal injection.

Viruses

rAAV-EF1α-DIO-hChR2-mCherry-WPRE-pA (for optogenetic stimulation experiments), rAAV-EF1α-DIO-hM3Dq-YFP-WPRE-pA and rAAV-EF1α-DIO-hM4Di-YFP-WPRE-pA (for chemogenetic approach experiments) were all purchased from BrainVTA Co. Ltd. (Wu Han, China).

Stereotaxic surgery

DAT-Cre mice were anesthetized with 70 mg/kg intraperitoneal (i.p.) sodium pentobarbital for stereotaxic surgeries. For optogenetic stimulation, the virus (rAAV-EF1α-DIO-hChR2-mCherry-WPRE-pA, 500 nl) was injected into the VTA (AP: -3.2; ML: -0.5; DV: −4.2 from the dura) unilaterally. For the chemogenetic approach, viruses (rAAV-EF1α-DIO-hM3Dq-YFP-WPRE-pA 500 nl or rAAV-EF1α-DIO-hM4Di-YFP-WPRE-pA 500 nl) were injected into the VTA (AP: -3.2; ML: ±0.5; DV: −4.2 from the dura) bilaterally. Then, the intracranial guide cannulas (Cat. No. 62003, 0.48 mm × 0.34 mm, O.D. × I.D., RWD Life Science, Shen Zhen,

China) were implanted into the VTA (AP: -3.2; ML: -0.5; DV: −3.7 from the dura) unilaterally for the optical fiber or into the NAc (AP: +1.6; ML: ±2.5; DV: −4.4 from the skull) bilaterally for microinjection. For microdialysis experiments, probe cannulas (MAB 10.8. IC, Sweden) were implanted into the NAc (AP: +1.6; ML: -0.8; DV: −3.8 from the skull). The coordinates above were all according to the mouse brain stereotaxic atlas of Franklin and Paxinos (2007). Then, the connector and cannulas were fixed to the skull with dental cement. Finally, all animals were allowed to recover for 2 weeks before experiments.

Apparatus

Apparatus for locomotion

Locomotion was measured in a locomotor activity monitoring chamber (400 mm × 400 mm × 350 mm; L × W × H) (JLBehv-LAG-9, Shanghai, China). The data were collected using AniLab ver. 2.2 for Loc software.

Apparatus for self-stimulation

The self-stimulation experiments were performed in operant test chambers (200 mm × 150 mm × 180 mm, L × W × H). Each chamber had two nose-poke holes (Aes-110, D.N. = 2 cm) located 4.5 cm above the floor (AniLab SuperState Version 4.0, AniLab Software & Instruments Co., Ltd., NingBo, China). Anilab 6.53 software was applied to schedule the experimental events and collect the data.

Behavioral experiments

Locomotor activity test

The DAT-Cre mice without virus injection and those expressing hM3Dq or hM4Di DREADDs were placed in locomotor detection chambers and allowed to habituate for half an hour per day for 3 d. On the test day, 20 min prior to placement in the chambers, mice were injected with CNO (1 mg/kg) intraperitoneally. The activity of the animals in the chambers was then recorded for 1.5 h. The data were collected using AniLab ver. 2.2 for Loc software.

Optic intracranial self-stimulation (oICSS) procedure

To determine the direct involvement of VTA DAergic neurons in reinforcement, we performed oICSS experiments using multiple frequencies or pulse durations of laser stimulation. Optic fibers were inserted into the mouse brain through the guide cannulas. The output power of the laser was adjusted to ~20 mW transmittance into the brain every day. Using a fixed ratio 1 (FR1) reinforcement schedule, an active poke resulted in laser stimulation (473 nm) with a 15-ms pulse at a series of frequencies (1, 5, 10, 20, 25, 50 and 65 Hz) for 3 s with 5-s cue light illumination and the house light off. When the cue was presented, the laser stimulation was followed by a 2-s timeout. Inactive pokes were counted but did not induce consequences. Each frequency was tested for a 10-min bin followed by the next frequency. During the laser stimulation and timeout period, additional active pokes were recorded but did not induce laser stimulation. To acquire the pulse-duration curve, we trained mice with a series of pulse durations (2, 5, 10, 15 and 20 ms) of laser stimulation in the same way. Then, to investigate the DA receptor involved in reinforcement, we examined the effect of the DA D1 receptor antagonist SCH23390 (0.2 mg/kg, i.p.) on the oICSS model.

In vivo microdialysis

General procedure

A probe (MAB 10.8.1. PES, Sweden) was inserted into the NAc of mice used for the microdialysis experiment, and the mice were then placed in the operant test chambers to minimize damage-induced DA release during experimentation. First, sterilized artificial cerebrospinal fluid (Compound Sodium Chloride Injection, Qidu Pharmaceutical Co., Ltd., China) was used as the dialysis buffer and was perfused through the probe (0.5 μl/min) for 30 min. Then, the perfusion speed was adjusted to 1.5 μl/min for 1 h and 2 μl/min for 2 h successively via a syringe pump (BASi, USA) before sample collection. To prevent DA degradation, we collected dialysis samples into tubes containing 15 μl stabilizer every 30 min. The stabilizer was prepared with 0.01 g Na_2EDTA, 220 μg vitamin C, 0.6 ml acetic acid and 100 ml ddH_2O. After 1.5 h of baseline collection, animals received continuous laser stimulation (3 h) with a 15-m pulse width at 20 Hz. At the same time, samples were collected every 30 min.

DA quantification

After collection, the dialysate of all samples was immediately measured using an electrochemical detection system (ESA). The DA mobile phase contained 80 mM $NaH_2PO_4 \cdot {}_2H_2O$, 0.74 mM sodium 1-octanesulfonate, 0.027 mM EDTA, 2 mM potassium chloride, and 10% methanol, pH 3.0. The high-performance liquid chromatography system (Sykam, German) consisted of the S2100 solvent delivery system, S5200 autosampler, Waters X Select @ HSS T3 chromatographic column (particle size: 2.5 μm, 2.1 × 50 mm; Waters, USA), Antec decade II electrochemical detector (Antec, Netherlands) and Clarity Chromatography Workstation (Clarity, Czech).

Histology

To confirm the expression of channelrhodopsin 2 (ChR2), hM3Dq-YFP or hM4Di-YFP on DAergic neurons, we anesthetized the mice with pentobarbitone sodium (70 mg/kg) and intracardially perfused them with ice-cold 0.9% saline followed by 4% paraformaldehyde to examine the colocalization of tyrosine hydroxylase (TH) and DAT-Cre expression. Brain tissues were then transferred to 20% and 30% sucrose successively for dehydration at 4 °C. Brains were coronally sectioned at 30 μm for immunofluorescence to detect the colocalization of mCherry or YFP and TH in the VTA. Brain sections containing VTA were blocked with 10% normal goat serum (NGS) and 0.3% Triton X-100 phosphate buffer for 2 h at room temperature. Immunofluorescence was then performed using a rabbit anti-TH monoclonal antibody (1:500; Abcam; ab6211) at 4 °C for 24 h. After the tissue was washed, the sections were further incubated with the secondary antibody for colocation of mCherry and TH: goat anti-rabbit Alexa Fluor 488 IgG (1:200; ZSGB-BIO; ZF-0511) in 10% NGS and 0.3% Triton X-100 phosphate buffer for 1.5 h at room temperature. For the colocation of YFP and TH, the sections were incubated with the secondary antibody donkey anti-rabbit Alexa Fluor 405 IgG (1:200; Abcam; ab175649) in 10% NGS and 0.3% Triton X-100 phosphate buffer for 1.5 h at room temperature. After incubation, brain sections were washed, mounted, and coverslipped. Finally, fluorescent images were obtained with a confocal microscope (Olympus, Japan).

Statistical analyses

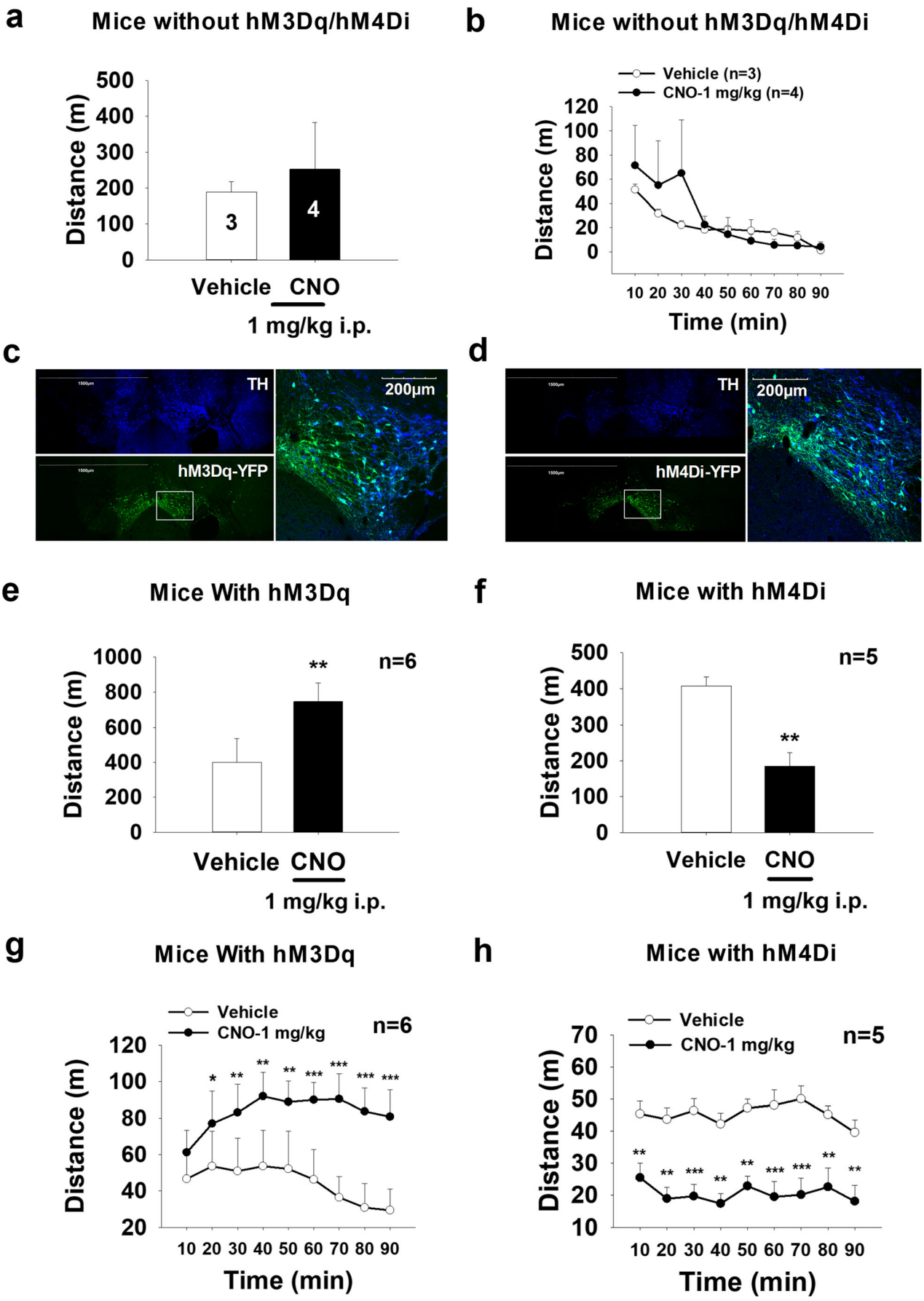
All data are presented as the mean ± SEMs. The motor activity of mice induced by the chemogenetic approach was analyzed by t-test. One-way repeated measures (RM) ANOVA was used to analyze the frequency or pulse-duration curve in the oICSS model and the change in the DA level in the microdialysis experiments. Two-way RM ANOVA was used to analyze the effect of SCH23390 on the frequency curve. Individual group comparisons in one-way or two-way ANOVA were performed with the Bonferroni test. The accepted level of significance for all tests was $P < 0.05$.

Results

VTA DAergic neurons modulated motor activity

Locomotion detection allows for the immediate and direct examination of motor activity. To investigate the relationship between DAergic neurons and motor activity, we examined the locomotion of mice when VTA DAergic neurons were specifically excited or inhibited using a chemogenetic approach. Mice were placed in the locomotor detection chamber and allowed to habituate for half an hour per day for 3 d. On the test day, mice were injected with CNO (1 mg/kg) or vehicle intraperitoneally 20 min before testing. Then, the distance mice traveled in 1.5 h and every 10 min were recorded. To exclude the effect of CNO itself on mouse locomotion, DAT-Cre mice not expressing hM3Dq or hM4Di in the VTA were injected with CNO or vehicle. In this group, CNO did not affect the motor activity of mice throughout the test compared to that with vehicle injection (Fig. 1a, b; Fig. 1a: t-test, $t = -0.470$, $P > 0.05$; Fig. 1b: two-way RM ANOVA: treatment main effect: $F_{1,\ 5} = 0.221$, $P = 0.658$; time main effect: $F_{8,\ 40} = 2.793$, $^{*}P < 0.05$; interaction: treatment × time: $F_{8,\ 40} = 0.570$, $P = 0.796$). Then, the experiment was performed with DAT-Cre mice expressing hM3Dq or hM4Di in VTA DAergic neurons. The colocalization of hM3Dq-YFP or hM4Di-YFP with TH is shown in Fig. 1c, d. In the hM3Dq group, compared with vehicle injection, CNO-induced excitation of VTA DAergic neurons significantly enhanced total locomotion (Fig. 1e: paired t-test, $t = -5.403$, $^{**}P < 0.01$). In addition, the distance traveled every 10 min showed that CNO persistently elevated the motor activity during the test (Fig. 1g; two-way RM ANOVA: treatment main effect: $F_{1,\ 5} = 29.194$, $^{**}P < 0.01$; time main effect: $F_{8,\ 40} = 1.309$, $P = 0.267$; interaction: treatment×time: $F_{8,\ 40} = 2.169$, $P = 0.051$). Individual group comparisons by Bonferroni test revealed a significant increase in locomotion induced by CNO injection compared to that observed with vehicle injection (10–90 min: $t = 1.289$, $P = 0.210$; $t = 2.081$, $^{*}P < 0.05$; $t = 2.857$, $^{**}P < 0.01$; $t = 3.402$, $^{**}P < 0.01$; $t = 3.258$, $^{**}P < 0.01$; $t = 3.878$, $^{***}P < 0.001$; $t = 4.793$, $^{***}P < 0.001$; $t = 4.689$, $^{***}P < 0.001$; $t = 4.562$, $^{***}P < 0.001$). In contrast, in

Fig. 1 Motor activity was modulated by VTA DAergic neuron activity. **a** CNO itself had no effects on total locomotion of mice without expressing hM3Dq or hM4Di ($n = 4$) compared to vehicle ($n = 3$), assessed by t test ($P > 0.05$). **b** The locomotion of mice without hM3Dq or hM4Di expression in 10-min bins was not affected by CNO injection (n = 4) compared to that with vehicle injection ($n = 3$), assessed by two-way RM ANOVA (treatment main effect: $F_{1,\ 5} = 0.221$, $P > 0.05$). **c** The expression of hM3Dq-YFP in the VTA. DAergic neurons labeled by TH (left, top). The expression of hM3Dq-YFP (left, bottom). Colocalization of hM3Dq-YFP and TH (right). **d** The expression of hM4Di-YFP in the VTA. DAergic neurons labeled by TH (left, top). The expression of hM4Di-YFP (left, bottom). Colocalization of hM4Di-YFP and TH (right). **e** hM3Dq-CNO-induced excitation of VTA DAergic neurons enhanced the locomotor activity of mice compared to vehicle injection ($n = 6$), assessed by paired t-test ($^{**}P < 0.01$). **f** hM4Di-CNO-induced inhibition of VTA DAergic neurons depressed the locomotor activity of mice compared to vehicle injection ($n = 5$), assessed by paired t-test ($^{**}P < 0.01$). **g** Compared to vehicle, CNO elevated the motor activity of mice with hM3Dq throughout the test (n = 6), assessed by two-way RM ANOVA followed by Bonferroni test ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). **h** Compared to vehicle, CNO persistently reduced the locomotion of mice with hM4Di during the test (n = 5), assessed by two-way RM ANOVA followed by Bonferroni test ($^{**}P < 0.01$, $^{***}P < 0.001$)


a
Mice without hM3Dq/hM4Di
Distance (m)
500
400
300
200
100
0
3
4
Vehicle CNO
1 mg/kg i.p.
b
Mice without hM3Dq/hM4Di
Vehicle (n=3)
CNO-1 mg/kg (n=4)
Distance (m)
120
100
80
60
40
20
0
10 20 30 40 50 60 70 80 90
Time (min)
c
TH
200μm
hM3Dq-YFP
d
TH
200μm
hM4Di-YFP
e
Mice With hM3Dq
Distance (m)
1000
800
600
400
200
0
**
n=6
Vehicle CNO
1 mg/kg i.p.
f
Mice with hM4Di
Distance (m)
500
400
300
200
100
0
n=5
**
Vehicle CNO
1 mg/kg i.p.
g
Mice With hM3Dq
Vehicle
CNO-1 mg/kg
n=6
Distance (m)
120
100
80
60
40
20
* ** ** ** *** *** ***
10 20 30 40 50 60 70 80 90
Time (min)
h
Mice with hM4Di
Vehicle
CNO-1 mg/kg
n=5
Distance (m)
70
60
50
40
30
20
10
** ** *** ** ** *** ** **
10 20 30 40 50 60 70 80 90
Time (min)

the hM4Di group, CNO-induced inhibition of VTA DAergic neurons significantly decreased the total locomotion compared with vehicle injection (Fig. 1f; paired t-test, $t = 5.571$, $^{**}P < 0.01$). Furthermore, the distance traveled every 10 min showed that CNO persistently depressed motor activity during the test (Fig. 1h; two-way RM ANOVA: treatment main effect: $F_{1,\ 4} = 31.040$, $^{**}P < 0.01$; time main effect: $F_{8,\ 32} = 0.995$, $P = 0.459$; interaction: treatment×time: $F_{8,\ 32} = 0.812$, $P = 0.597$). Individual group comparisons by Bonferroni test revealed a significant reduction in locomotion induced by CNO injection compared to those observed with vehicle injection (10–90 min: $t = 3.982$, $^{**}P < 0.01$; $t = 4.791$, $^{**}P < 0.01$; $t = 5.195$, $^{***}P < 0.001$; $t = 4.410$, $^{**}P < 0.01$; $t = 4.686$, $^{**}P < 0.01$; $t = 5.047$, $^{***}P < 0.001$; $t = 5.390$, $^{***}P < 0.001$; $t = 4.461$, $^{**}P < 0.01$; $t = 3.833$, $^{**}P < 0.01$). These results suggest that the activity of VTA DAergic neurons directly modulates motor activity.

Stimulation of VTA DAergic neurons regulated reinforcement through D1 receptor

Specific stimulation of VTA DAergic neurons directly induced reinforcement

To clarify whether DAergic neurons directly regulate reinforcement, laser stimulation of multiple frequencies and pulse durations in the oICSS model were used. The photosensitive protein ChR2 was expressed in VTA DAergic neurons of DAT-Cre mice before the oICSS experiment. The colocalization of ChR2-mCherry and TH is shown in Fig. 2a. First, the pulse duration of laser stimulation was fixed at 15 ms. Mice were placed in the operant test chambers and trained to poke for the photostimulation delivered into the VTA at multiple frequencies (1 Hz, 5 Hz, 10 Hz, 20 Hz, 25 Hz, 50 Hz and 65 Hz). The number of active pokes (Fig. 2b) but not inactive pokes (Fig. 2c) increased according to the increase in frequency (Fig. 2b: active poke: one-way RM ANOVA: frequency main effect: $F_{6,\ 36} = 23.489$, $^{***}P < 0.001$; Fig. 2c: inactive poke: one-way RM ANOVA: frequency main effect: $F_{6,\ 36} = 0.948$, $P = 0.473$). Then, the frequency of laser stimulation was fixed at 20 Hz. Mice were trained to poke for photostimulation of multiple pulse durations (2 ms, 5 ms, 10 ms, 15 ms and 20 ms). Similarly, the number of active pokes (Fig. 2d) but not inactive pokes (Fig. 2e) increased in a pulse duration-dependent manner (Fig. 2d: active poke: one-way RM ANOVA: pulse duration main effect: $F_{4,\ 16} = 19.687$, $^{***}P < 0.001$; Fig. 2e: inactive poke: one-way RM ANOVA: pulse duration main effect: $F_{4,\ 16} = 2.086$, $P = 0.130$). These results indicate that the intensity of reinforcement positively correlates with the activity of VTA DAergic neurons.

Specific stimulation of VTA DAergic neurons increased the level of DA in the NAc

The results above demonstrate that DAergic neurons in the VTA positively regulate reinforcement. Then, to determine whether the reinforcement behavior was correlated with elevations in DA induced by VTA DAergic neuron stimulation, we investigated the concentration of DA in the NAc through microdialysis experiments. Figure 3a shows the microdialysis schedule and laser stimulation parameters, and Fig. 3b shows the schematic diagram depicting VTA DAergic optogenetic stimulation and microdialysis in the NAc. Laser stimulation with a pulse width of 15 ms and frequency of 20 Hz significantly enhanced the concentration of DA in the NAc compared with the baseline (The averages of the baseline and the points boxed in Fig. 3c were analyzed by one-way RM ANOVA: time main effect: $F_{4,\ 8} = 5.082$, $^{*}P < 0.05$).

The reinforcement induced by VTA DAergic neuron-specific stimulation was decreased by DA D1 receptor antagonist

Furthermore, we also researched the receptor mechanism underlying the reinforcement. As shown in Fig. 4a, systemic administration of the D1 receptor antagonist SCH23390 (0.2 mg/kg, i.p.) shifted the frequency curve down compared with the vehicle in the oICSS model (active poke: two-way RM ANOVA: treatment main effect: $F_{1,\ 6} = 42.064$, $^{***}P < 0.001$; frequency main effect: $F_{6,\ 36} = 14.069$, $^{***}P < 0.001$; interaction: treatment × frequency: $F_{6,\ 36} = 10.482$, $^{***}P < 0.001$). Individual group comparisons assessed by Bonferroni test revealed a significant reduction in the number of active pokes with antagonist administration compared to that with vehicle: 1–65 Hz: $t = 1.808$, $P = 0.094$; $t = 3.537$, $^{**}P < 0.01$; $t = 4.451$, $^{***}P < 0.001$; $t = 6.025$, $^{***}P < 0.001$; $t = 5.818$, $^{***}P < 0.001$; $t = 6.984$, $^{***}P < 0.01$; $t = 7.839$, $^{***}P < 0.001$). As demonstrated in Fig. 4b, the number of inactive pokes was not affected by SCH23390 (two-way RM ANOVA: treatment main effect: $F_{1,\ 6} = 1.299$, $P = 0.298$; frequency main effect: $F_{6,\ 36} = 1.524$, $P = 0.198$; interaction: treatment × frequency: $F_{6,\ 36} = 1.677$, $P = 0.155$).

Discussion

Although the level of DA in the brain is often associated with reward, including motor activity and reinforcement, whether the activity of DA neurons in the VTA directly modulates movement and reinforcement remained ambiguous. In this study, selective activation or inhibition of VTA DA neurons with chemogenetic methods induced drastic hyperactivity or hypoactivity. In addition, selective activation of VTA DA neurons using with optical manipulation established reinforcement behavior in a frequency- and pulse duration-dependent

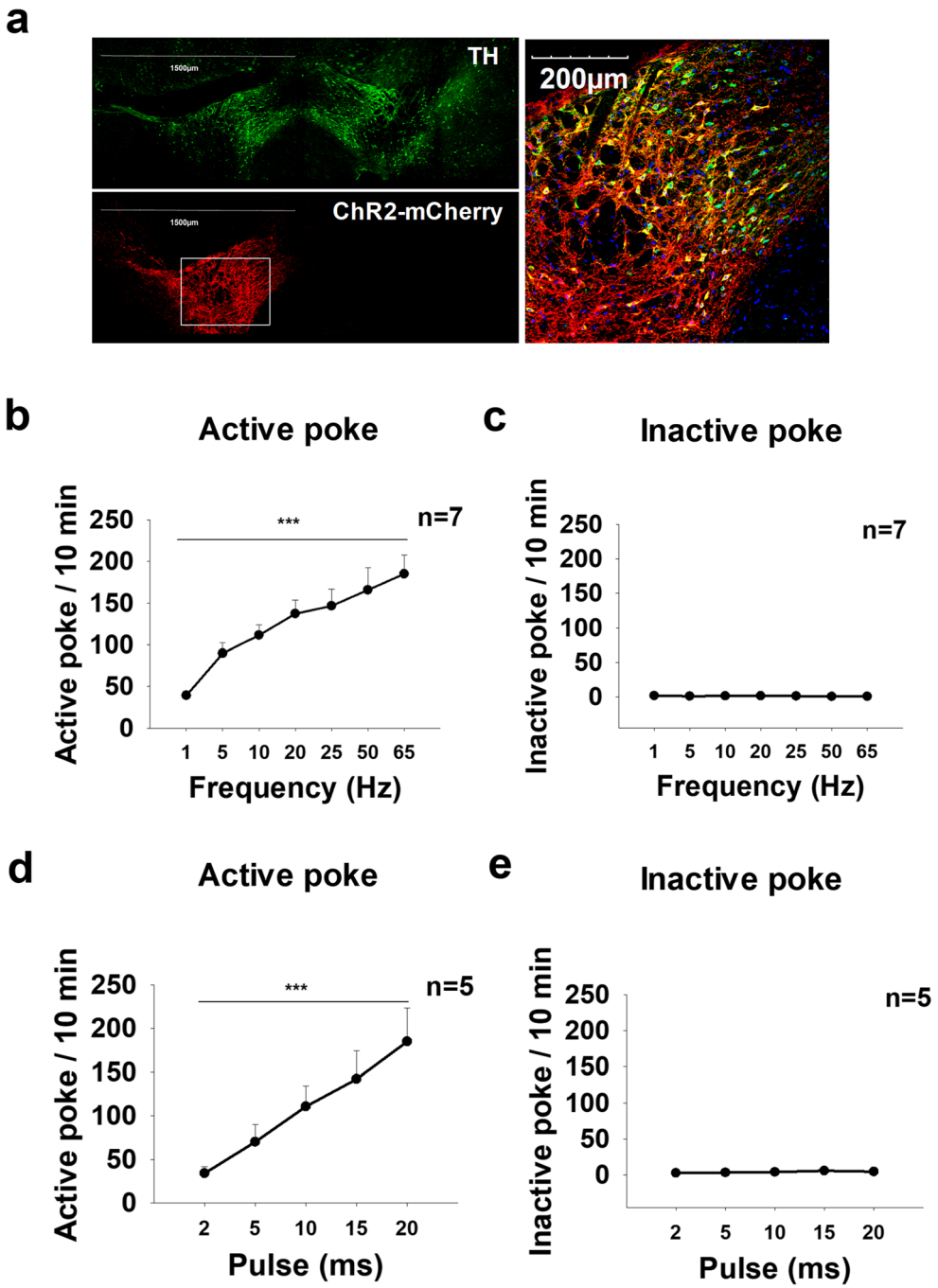


Fig. 2 Reinforcement was modulated by VTA DAergic neuron activity. **a** The expression of ChR2-mCherry in the VTA. DAergic neurons labeled by TH (left, top). The expression of ChR2-mCherry (left, bottom). Colocalization of ChR2-mCherry and TH (right). **b, c** The frequency curve established by optogenetic excitation of VTA DAergic neurons with multiple frequencies. The number of active pokes but not inactive pokes increased in a frequency-dependent manner ($n = 7$). Active pokes: one-way RM ANOVA: frequency main effect: $F_{6, 36} = 23.489$, $^{***}P < 0.001$; inactive pokes: one-way RM ANOVA: frequency main effect: $F_{6, 36} = 0.948$, $P > 0.05$. **d, e** The pulse-duration curve established by optogenetic excitation of VTA DAergic neurons with multiple pulse durations. The number of active pokes but not inactive pokes increased in a pulse duration-dependent manner (n = 5). Active pokes: one-way RM ANOVA: pulse duration main effect: $F_{4, 16} = 19.687$, $^{***}P < 0.001$; inactive pokes: one-way RM ANOVA: pulse duration main effect: $F_{4, 16} = 2.086$, $P > 0.05$

manner, and the reinforcement behavior was regulated through DA receptors in the NAc.

Analysis of locomotion allows for the visualization of the rewarding effect of stimuli to animals. Hyperactivity is induced by addictive drugs targeting the DA system and genetic knockout or knockdown of DAT, which is usually interpreted to increase brain DA levels (Salahpour et al. 2007; Spielewoy et al. 2000). However, the above predisposing factors may not specifically relate to VTA DA signaling. The blockade of DA reuptake by drugs and the genetic knockout or knockdown of DAT increases DA levels all over the brain, potentially alteration the formation of neural circuits during development. In our study, hyperactivity was induced by selective excitation of DAergic neurons in the VTA, which also induced an increase in DA concentration assessed by microdialysis. The present results are similar to those described in previous research and are consistent with Wang's study in which DAergic neurons were activated by chemogenetic methods (Wang et al. 2013). Nevertheless, Wang's studies did not distinguish between DA cells in the VTA and SNc. Furthermore, the influence of inhibition of VTA DAergic neurons on locomotion has not been investigated in previous research. Interestingly,

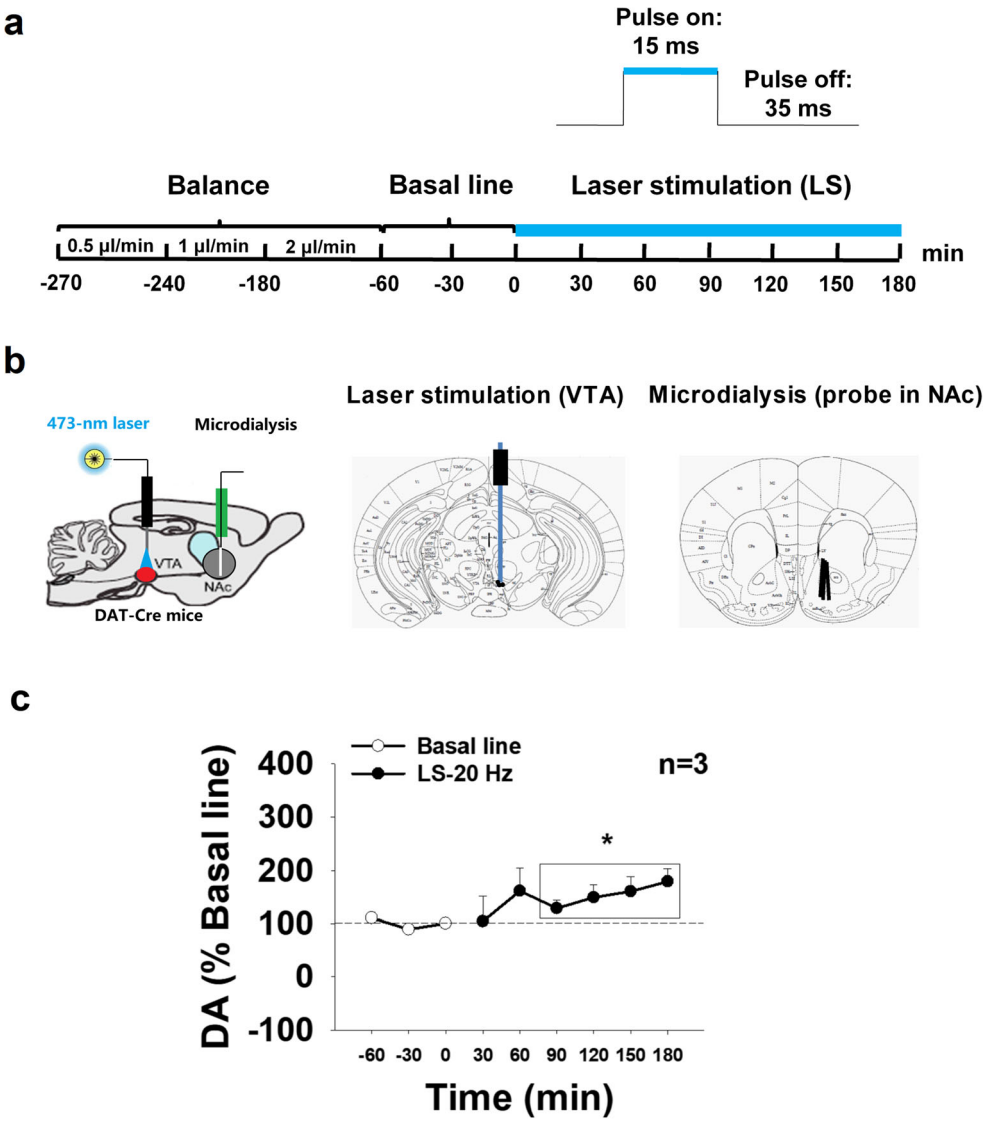


Fig. 3 The concentration of DA in the NAc was enhanced by optogenetic excitation of VTA DAergic neurons. **a** The microdialysis schedule and laser stimulation parameters. Sterilized artificial cerebrospinal fluid was perfused through the probe at a speed of 0.5 μl/min for 30 min, 1 μl/min for 1 h and 2 μl/min for 2 h before sample collection to minimize damage-induced DA release during experimentation. Then, samples were collected every 30 min at the stable speed of 2 μl/min during baseline phase and laser stimulation phase. **b** The schematic diagram showing the optogenetic stimulation of VTA DAergic neurons and microdialysis in the NAc. **c** The concentration of DA was significantly enhanced by optogenetic excitation of VTA DAergic neurons (n = 3), assessed by one-way RM ANOVA ($F_{4,\ 8}$ = 5.082, $^{*}P < 0.05$)

DA system dysfunction and hypoactivity induce hypoactive and hyperactive locomotion. Parkinson's disease is characterized as a movement disorder associated with DAergic neuron injury and low DA transmission. Our observed effects of DAergic neuron inhibition are consistent with these theories. However, the core pathogeny of attention deficit/hyperactivity disorder (ADHD) is a hypofunctional DA system that induces hyperactive locomotion. Nevertheless, this seeming contradiction does not conflict with our result. The abnormally low tonic DA level in ADHD causes a relative upregulation of the efficiency of the phasic response of DAergic neurons, leading to hypersensitivity to environmental stimuli (Sikstrom and Soderlund 2007). However, in the present study, chemogenetic inactivation of VTA DAergic neurons inhibited both tonic and phasic responses, thereby decreasing the DA level in the NAc and reducing motor activity. Above all, our findings confirm and extend our understanding of the direct relationship between DAergic neurons and the regulation of motor activity.

ICSS is a classic positive reinforcement model based on the reward system. In our previous study and other studies (Pascoli et al. 2015; Rossi et al. 2013; Steinberg and Janak 2013; Witten et al. 2011), stimulation of VTA DAergic neurons was able to cause self-stimulation behavior and induce a learning process. However, other studies presented a visual cue along with the nose poke-initiated DAergic neuron stimulation. Therefore, whether the optical stimulation of DAergic neurons directly reinforces the instrumental action or acts as a conditioned reinforcer via its association with the cue is unclear. In the present research, we used multiple frequencies and pulse durations of laser stimulation under the same visual cue and showed that the

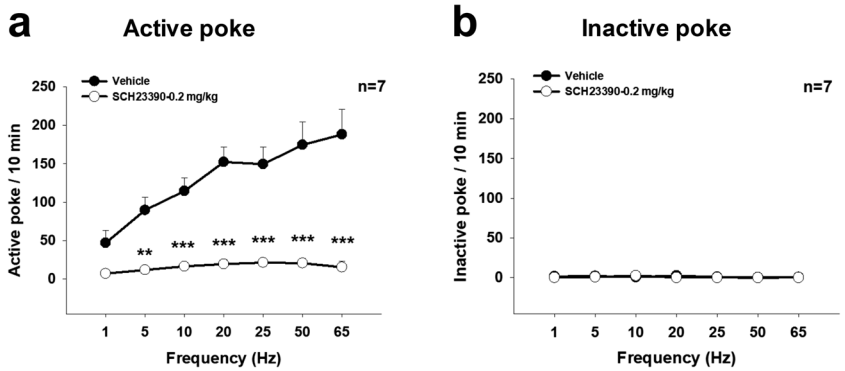


Fig. 4 DA D1 receptor was involved in reinforcement. **a** The DA D1 receptor antagonist SCH23390 significantly depressed the frequency curve of active pokes compared to vehicle (n = 7), assessed by two-way RM ANOVA followed by Bonferroni test ($^{**}P < 0.01$, $^{***}P < 0.001$). **b** SCH23390 had no effect on inactive pokes compared to vehicle (n = 7), assessed by two-way RM ANOVA (treatment main effect: $F_{1,\ 6} = 1.299$, $P > 0.05$; frequency main effect: $F_{6,\ 36} = 1.524$, $P > 0.05$; interaction: treatment × frequency: $F_{6,\ 36} = 1.677$, $P > 0.05$)

active pokes were enhanced in a frequency and pulse duration-dependent manner. Although similar findings have been reported with the electrical self-stimulation of the VTA, the previous experiments did not specifically activate the DAergic neurons in the VTA (Depoortere et al. 1999). In addition, we also measured the DA concentration in the NAc with optogenetic stimulation of VTA DAergic neurons at a high frequency (20 Hz), resulting in DA release faster than its uptake. In this condition, an elevated concentration of DA in the NAc was observed, potentially reaching a level sufficient for generating reward (Bass et al. 2010; Robinson and Wightman 2004). Furthermore, systematic injection of the DA D1R antagonist shifted the frequency curve down and decreased the number of active pokes but not inactive pokes, indicative of a reduction in reinforcement behavior (Steinberg et al. 2014). Additive drugs increase DA release in the mesocorticolimbic system associated with reinforcement, motivation and goal-directed behavior (Di Chiara and Imperato 1988; Horvitz 2000; Schultz 1998; Wise 2004; Zink et al. 2003), while DA receptor antagonists inhibit the reinforcement induced by addictive drugs. Therefore, our results are consistent with these studies. Together, our results demonstrate that manipulation of DAergic neurons in the VTA directly regulates the reinforcement process.

In conclusion, our study used chemogenetic and optogenetic approaches to demonstrate that VTA DAergic neurons regulate motor activity and reinforcement, which are related to reward. Because the VTA DAergic system is widely involved in diverse arrays of cognitive processes, chemogenetic and optogenetic approaches can be used to study the effects of DAergic intervention on animal performance in other behavioral tests, thereby contributing to a deepening of our understanding of the underlying mechanisms of reward.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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