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ORIGINAL ARTICLE

Medium spiny neurons of the anterior dorsomedial striatum mediate reversal learning in a cell-type-dependent manner

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Abstract

The striatum has been implicated in the regulation of cognitive flexibility. Abnormalities in the anterior dorsomedial striatum (aDMS) are revealed in many mental disorders in which cognitive inflexibility is commonly observed. However, it remains poorly understood whether the aDMS plays a special role in flexible cognitive control and what the regulation pattern is in different neuronal populations. Based on the reversal learning task in mice, we showed that optogenetic activation in dopamine receptor 1-expressing medium spiny neurons (D1R-MSNs) of the aDMS impaired flexibility; meanwhile, suppressing these neurons facilitated behavioral performance. Conversely, D2R-MSN activation accelerated reversal learning, but it induced no change through neuronal suppression. The acquisition and retention of discrimination learning were unaffected by the manipulation of any type of MSN. Through bi-direct optogenetic modulation in D1R-MSNs of the same subject in a serial reversal learning task, we further revealed the function of D1R-MSNs during different stages of reversal learning, where inhibiting and exciting the same group of neurons reduced perseverative errors and increased regressive errors. Following D1R- and D2R-MSN activation in the aDMS, neuronal activity of the mediodorsal thalamus decreased and increased, respectively, in parallel with behavioral impairment and facilitation, but not as a direct result of the activation of the striatal MSNs. We propose that D1R- and D2R-MSN sub-populations in the aDMS exert opposing functions in cognitive flexibility regulation, with more important and complex roles of D1R-MSNs involved. Mental disorders with cognitive flexibility problems may feature an underlying functional imbalance in the aDMS' two types of neurons.

Keywords Anterior dorsomedial striatum · Behavioral flexibility · Optogenetics · Medium spiny neurons · Dopamine receptors

Introduction

Cognitive flexibility is an essential executive function that enables individuals to switch between strategies to cope with changing environments (Moore and Malinowski 2009). Its deficits are commonly observed in many mental disorders, such as Parkinson's disease, schizophrenia, drug addiction, and autism (McCracken and Grace 2013; Orellana and Slachevsky 2013; Darvas et al. 2014; D'Cruz et al. 2016). Animal and human studies have revealed that the caudate plays a crucial role in the regulation of cognitive flexibility. For example, caudate inactivation impaired reversal learning in rats and monkeys (Ragozzino 2007; Clarke et al. 2008), and the caudate showed greater responses to reversal errors relative to acquisition errors (Ghahremani et al. 2010). Among the subparts of the caudate, the head might be more specifically related to flexible learning processes (Ghahremani et al. 2010; Ruge and Wolfensteller 2016). However, how the caudate head regulates cognitive flexibility remains unclear. Meanwhile, several clinical studies imply that damaged function in the caudate head may underlie mental or psychiatric disorders with impaired cognitive flexibility (Rinne et al. 1989; Lisanby et al. 1993; Levitt et al. 2003; Sweitzer et al. 2014; Tessa et al. 2014). Thus, insight into the regulation pattern of the caudate head in cognitive flexibility

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is valuable for understanding and monitoring the development of these disorders.

In humans, the caudate head is known to receive projections from neurons of the medial prefrontal cortex (mPFC) (Rolls et al. 1983). The corresponding projections can be observed from the mPFC targeting the anterior dorsomedial striatum (aDMS) in rodents (Berendse et al. 1992; Voorn et al. 2004). Our study revealed the link between cognitive inflexibility and high-dose exposure to morphine and hyperactivity in the mPFC–striatum circuit underlying this cognitive dysfunction (Piao et al. 2017). A similar implication for mPFC function was reported in a previous study (Graybeal et al. 2011). Furthermore, the mediodorsal (MD) thalamus, which is a significant component of the mPFC–striatum–globus pallidus–MD thalamus circuit (Mitchell 2015), and it is reportedly involved in flexible goal-directed behaviors (Parnaudeau et al. 2015). These previous findings further indicated that the aDMS, as well as the mPFC–aDMS–globus pallidus–MD thalamus circuit, might play an important role in modulating cognitive flexibility.

Striatum is composed of 95% medium spiny neurons (MSNs). These projection neurons form two major output pathways based on their projection targets, in which information can be relayed back to the cortex via the thalamus. The dopamine 1 receptors (D1R) are preferentially localized on MSNs that project to the substantia nigra pars reticulata (SNR) and the internal globus pallidus (GPi), which is referred to as the direct striatonigral pathway. On the other hand, the dopamine 2 receptors (D2R) are preferentially localized on MSNs that project to the SNR and GPi though a multi-synaptic pathway within the basal ganglia. These MSNs project to the external globus pallidus (GPe) and the subthalamic nucleus (STN) before reaching the SNR/GPi, which is referred to as the indirect striatopallidal pathway (Gerfen et al. 1990). In traditional models, the function of the striatum was highlighted in the proposed dichotomy of striatal MSN subtypes, where D1R-MSNs and D2R-MSNs would have an opposite but balancing role in the control of motor behavior (Albin et al. 1989). Additionally, the dissociated roles of D1R- and D2R-MSNs in the striatum were also suggested to regulate reward and aversive behaviors (Hikida et al. 2010). Based on pharmacological methods, some studies identified a role for D1R signaling in cognitive flexibility (McLean et al. 2009; Bestmann et al. 2015), while others showed the importance of D2R signaling (Cools et al. 2007b; Haluk and Floresco 2009). However, evidence of how striatal D1R- and D2R-MSNs modulate cognitive flexibility is absent. Combined with the previous implication that D2R-MSNs may predominate under basal conditions while the D1R-MSNs becomes more dominant when the reward-associated dopamine release increases (Nagai et al. 2016), we hypothesized that D1R- and D2R-MSNs in the aDMS and their segregated mediation in the direct/indirect pathways played opposite roles in the regulation of cognitive flexibility with the dominant function of D1R-MSNs on it.

To address these concerns, we used an optogenetic approach to elucidate the roles of the D1R direct and D2R indirect pathways in the aDMS in mediating cognitive flexibility. To get the mice with channelrhodopsin-2 (ChR2) or halorhodopsin (NpHR)-specific expressions in D1R- or D2R-MSNs, the transgenic mice carrying the D1R-Cre or adenosine A2A receptor (A2R)-Cre were crossed with Cre-dependent ChR Ai32/NpHR Ai39 mice. The A2R-Cre line is an alternative transgenic mouse line that functions comparable to D2R-Cre (Durieux et al. 2009). Several studies reported that they were co-localized and could form A2R-D2R heteromers (Sahlholm et al. 2018). In striatal MSNs, the A2R tightly interacts structurally and functionally with the D2R (Schiffmann et al. 2007), but it is not supposed to not be expressed in striatal cholinergic interneurons (Schiffmann and Vanderhaeghen 1993). Here, we found that activating D1R-MSNs and D2R-MSNs, respectively, disrupted and facilitated a reversal performance without affecting the acquisition or retention of discrimination learning. The MD thalamus was regulated directly and indirectly by aDMS neurons, the activities of which were identified in a functional pattern by immunohistochemistry following light stimulation-modulated behaviors and only light stimulation. Results revealed that neuronal activities in the MD thalamus were down-regulated and up-regulated in parallel with the reversal learning performance.

Materials and methods

Subjects

In the present study, bacterial artificial chromosome (BAC) transgenic mouse lines that express Cre recombinase under control of the D1R (https://www.mmrrc.org/catalog/sds.php?mmrrc_id=30989) and A2R (https://www.mmrrc.org/catalog/sds.php?mmrrc_id=36158) regulatory elements were used. D1R-Cre or A2R-Cre mice were crossed with mice in which the expression of the ChR2 (https://www.jax.org/strain/012569) or NpHR gene (https://www.jax.org/strain/014539) from an ubiquitously active promoter is prevented by a *loxP*-flanked stop cassette (Madisen et al. 2012), leading to double transgenic D1R-Cre/+ or A2R-Cre/+; ChR2/+ or NpHR/+ mice that selectively express ChR2/NpHR in D1R/D2R neurons (Fig. 1). Animals used in the study were male mice at ~ 10 weeks of age weighing ~ 30 g. All the mice were housed in a 12-h light–dark cycle (lights on at 7:00 a.m.) room. All procedures were approved by the Institutional Review Board of the Institute of Psychology, Chinese Academy of Sciences, and they complied with the National Institutes of Health Guide for Care and Use of

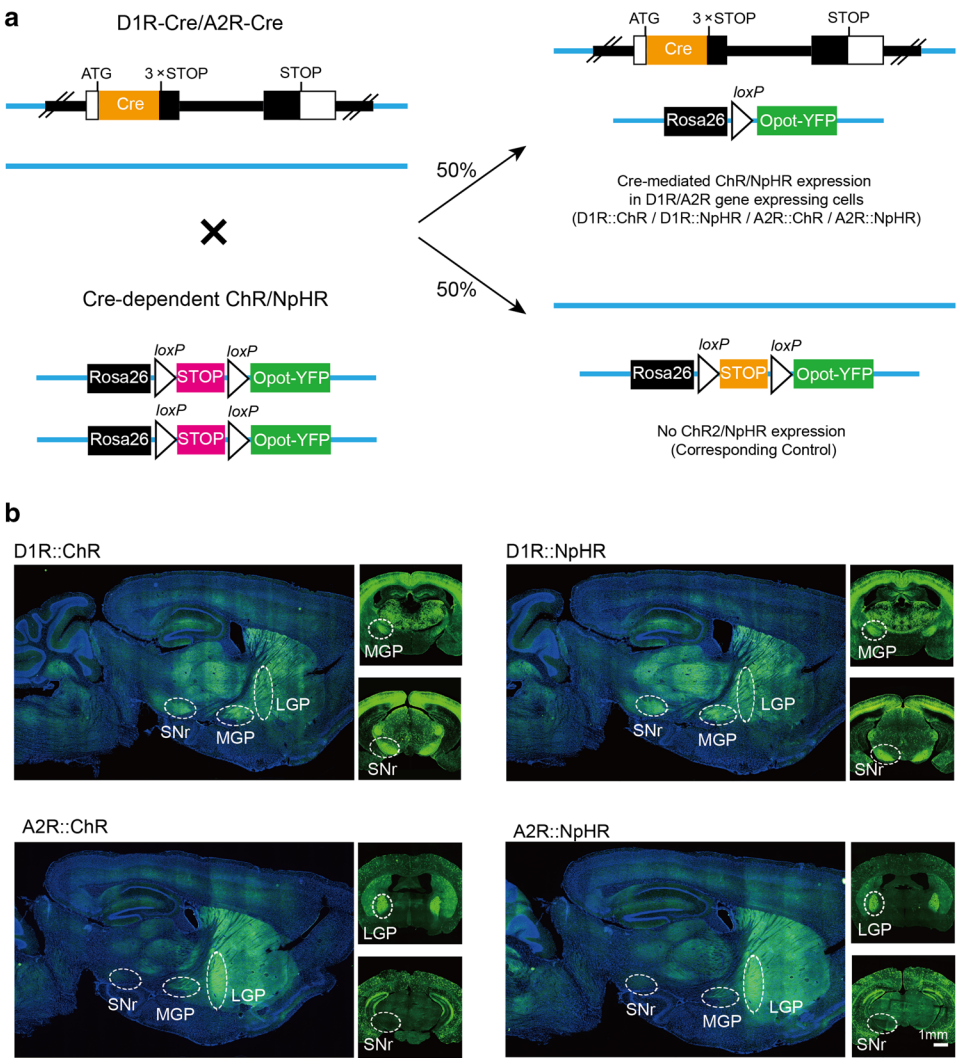


Fig. 1 By crossing D1R-cre or A2R-cre mice with Cre-dependent ChR Ai32 or NpHR Ai39 mice (**a**), we got selected offspring that could be specifically optogenetically manipulated in D1R-MSNs or D2R-MSNs (D1R::ChR/D1R::NpHR and A2R::ChR/A2R::NpHR). The sagittal and coronal views of the optogenetic proteins in the crossing mice brains (optogenetic proteins: green, DAPI: blue); **b** the proteins were mainly distributed on the striatal direct pathway (D1R::ChR/D1R::NpHR) or the indirect pathway (A2R::ChR/A2R::NpHR)

Laboratory Animals. Ten experiments were conducted in the present study, and the corresponding information, including mice cohorts, manipulations, and assessments, is shown in Table 1.

Virus preparation and stereotaxic injection

A double *loxP*-flanked inverted (DIO) strategy was used in ChR2 fused with mCherry or mCherry alone in Cre-expressing neurons. The constructs of DIO-ChR2-mCherry and DIO-mCherry were cloned into an adeno-associated virus (AAV) vector. The recombinant AAV vectors were serotyped with AAV2/8 coat proteins and packaged by Obio Technology Co., Ltd. (Shanghai, China). Genomic titers of AAVs were measured by quantitative real-time polymerase chain reaction (PCR) and adjusted to 1.0×10^{13} genomic copies/ml for stereotaxic injections.

Anesthetized [125–250 mg/kg avertin (2, 2, 2-tribromoethanol), i.p.] mice were injected into the aDMS bilaterally with a glass pipette at a flow rate of 0.15 µl/min. Coordinates used for the stereotaxic injections of the aDMS were AP + 1.0 mm, ML ± 1.5 mm, and DV − 3.0 mm. We injected 1 µl of the DIO-ChR2-mCherry virus into the left and right dorsomedial striata. The pipette was left in place for 5 min following the injection and then slowly removed. To allow time for viral expression, we housed animals for at least 2 weeks following injection before any experiments were initiated.

Optic fiber implantation and optogenetic stimulation

A plastic mount containing two fibers (105-µm core and 125-µm cladding) mounted in 1.25-mm zirconia ferrules was gently lowered and located into the brain and cemented in place for each fiber aiming at the aDMS (AP + 1.00 mm, ML ± 1.5 mm, DV − 3.00 mm) or MD thalamus (AP − 1.58 mm, ML − 0.40 mm, DV − 2.50 mm)

Table 1 List of experiments

Group	Figures	Manipulation	Mice (sample size)	End points
1	2b	473/589-nm light stimulation	D1R::ChR(3) D1R::NpHR(3) Control(3)	c-Fos immunostaining
2	2c	473/589-nm light stimulation	D1R::ChR(1) D1R::NpHR(1)	In vitro slice electro-physiology
3	2d–g 2h–k	473/589-nm light stimulation	D1R::ChR(6) and Control(7) D1R::NpHR(6) and Control(7)	Reversal learning task and open field test
4	3b–e 3f–i	473-nm light stimulation	A2R::ChR(8) and Control(8) A2R::NpHR(8) and Control(7)	Reversal learning task and open field test
5	4c–e	473/589-nm light stimulation	D1R::ChR::NpHR(8) and Control(8)	Reversal learning task
6	5b–e 6	473/589-nm light stimulation	D1R::ChR(8/5) and Control(8/4) D1R::NpHR(8/5) and Control(8/4) A2R::ChR(8/4) and Control(8/3) A2R:: NpHR(8/3) and Control(8/4)	Reversal learning task c-Fos immunostaining
7	7	473/589-nm light stimulation	D1R::ChR(5) and Control(3) A2R::ChR(5) and Control(3)	c-Fos immunostaining
8	8a	Viral injection	D1R-Cre(3)	Tracing imaging
9	8c, d	Viral injection and 473-nm light stimulation	D1R-Cre_ChR2(7) D1R-Cre_Control(7)	Reversal learning task and open field test
10	8e	Viral injection and 473-nm light stimulation	D1R-Cre_ChR2(6) D1R-Cre_Control(6)	c-Fos immunostaining

A summary of experiments reported in this manuscript is provided, with corresponding figure numbers

(1) A2R, adenosine A2A receptor; ChR, channelrhodopsin; D1R, dopamine 1 receptors; NpHR, halorhodopsin; (2) In Groups 1–7, the controls were the transgenic littermates expressing no ChR2/NpHR; in Groups 8–10, the controls were the D1R-Cre mice with AAV-DIO-mCherry injection

on either side (0.5 mm above the targeted positions). A blue laser (473 nm, 20 Hz, 5-ms stimulation duration per 5 s, 20-mW power) was used to activate targeted neurons, while a yellow laser (589 nm, 2900 ms constant stimulation with 100-ms intervals, 20-mW power) was used to inhibit targeted neurons.

Reversal learning task

The behavioral experiments were conducted in four operant chambers (23 cm × 17 cm × 20 cm; AniLab Software & Instruments Co., Ltd., Ningbo, China) that were enclosed in sound-attenuating boxes. Each chamber was fitted with two nose-poke holes, and a central liquid receptacle was located between them. Two yellow light-emitting diode lights (20 mW) were separately situated inside the nose-poke holes, and a white chamber light was located 10 cm above the right nose-poke hole. Solutions were delivered through a metal spout that was attached to a peristaltic pump with tubing that delivered fluid at a rate of 1.2 ml/min. The pumps were calibrated to dispense 0.02 ml (0.1 s) of solution into a liquid receptacle for each reinforcement. Following each reinforcement was a 15-s period, during which time any further nose pokes would get no more rewards.

Test preparation

Mice were weighed, singly housed, and handled by the experimenter during three alternate days before the formal procedure. To ensure the mice had enough motivation to get the sucrose rewards, 3 days before and throughout the whole experiment procedure, mice were food-restricted to maintain 85–90% of their baseline free-feeding body weight.

Habituation

For habituation to the apparatus, in the first 2 days, mice were simply put into habituation sessions. On day 1, mice were placed in the operant chambers and allowed to explore the chamber freely for 15 min with the nose-poke light off. Sucrose reinforcement was unavailable during this period. The mice were then trained to understand that the sucrose solution was available from the central liquid receptacle for 60 min. The fluid was delivered into the central liquid receptacle on a variable interval (VI) (10–50 s) schedule. When the fluid was dispensed with the running sound of the pump, the house light (i.e., conditioned stimulus) was turned on and remained illuminated for 4 s. In total, 20% sucrose (w/v) in tap water was used as the fluid reward. On day 2, 45-min fluid training was conducted after 5 min of chamber exploration.

Response discrimination

During the response discrimination phase, the mice had to learn the correct response at the nose-poke hole, which was picked randomly (Fig. 2a). One successful session consists of eight consecutive correct responses. The trials continued until the mice achieved the criterion (i.e., complete three sessions). The total number of trials and error trials conducted by each mouse was recorded.

Reversal learning

Twenty-four hours after the response discrimination phase, the mice were required to learn a reversal type of the response rule (Fig. 2a). The mice had to nose poke in the hole that was opposite that which they learned the preceding day. Thus, if a mouse had learned to nose poke in the left hole during the discrimination phase, then it had to nose poke in the right hole in the reversal learning phase instead, regardless of the position of the nose-poke light. The other aspects of the training were kept identical to the response discrimination training day. During the response discrimination and reversal learning phases, the light in the nose-poke hole was illuminated randomly prior to the start of each trial to serve as a distracting stimulus.

Each time when a mouse made an incorrect response, it would be scored as an error. All the errors conducted during the reversal learning phase could be categorized into two types: perseverative errors (PEs) and regressive errors [REs; Boulougouris et al. 2007, 2009; Haluk and Floresco 2009]. PEs reflected the impact of previously learned strategies on current strategies, which usually occurred in the early stage during reversal learning (Graybeal et al. 2011). Meanwhile, REs reflected the instability of maintaining new strategies, which usually occurred in the late stage during reversal (Graybeal et al. 2011). Rules for identifying PEs and REs from all errors of reversal can differ according to different equipment and programs. Based on previous studies (Bussey et al. 1997; Chudasama et al. 2001; Dickson et al. 2010, 2013) and modified by Floresco et al. (2009) and Zhang et al. (2012), accuracy was used as a measurement to distinguish PEs and REs in the present study. Accuracy was calculated from the 16th trial to the end of reversal (Nth trial). A_i (accuracy in the ith trial) was considered the sum of the number of correct trials from the $[(i-16)+1]$th trial to the ith trial divided by 16 and multiplied by 100%. Errors when the accuracy was $\leq 38\%$ were counted as PEs. Conversely, errors when the accuracy was $>38\%$ were counted as REs.

Serial reversal learning task

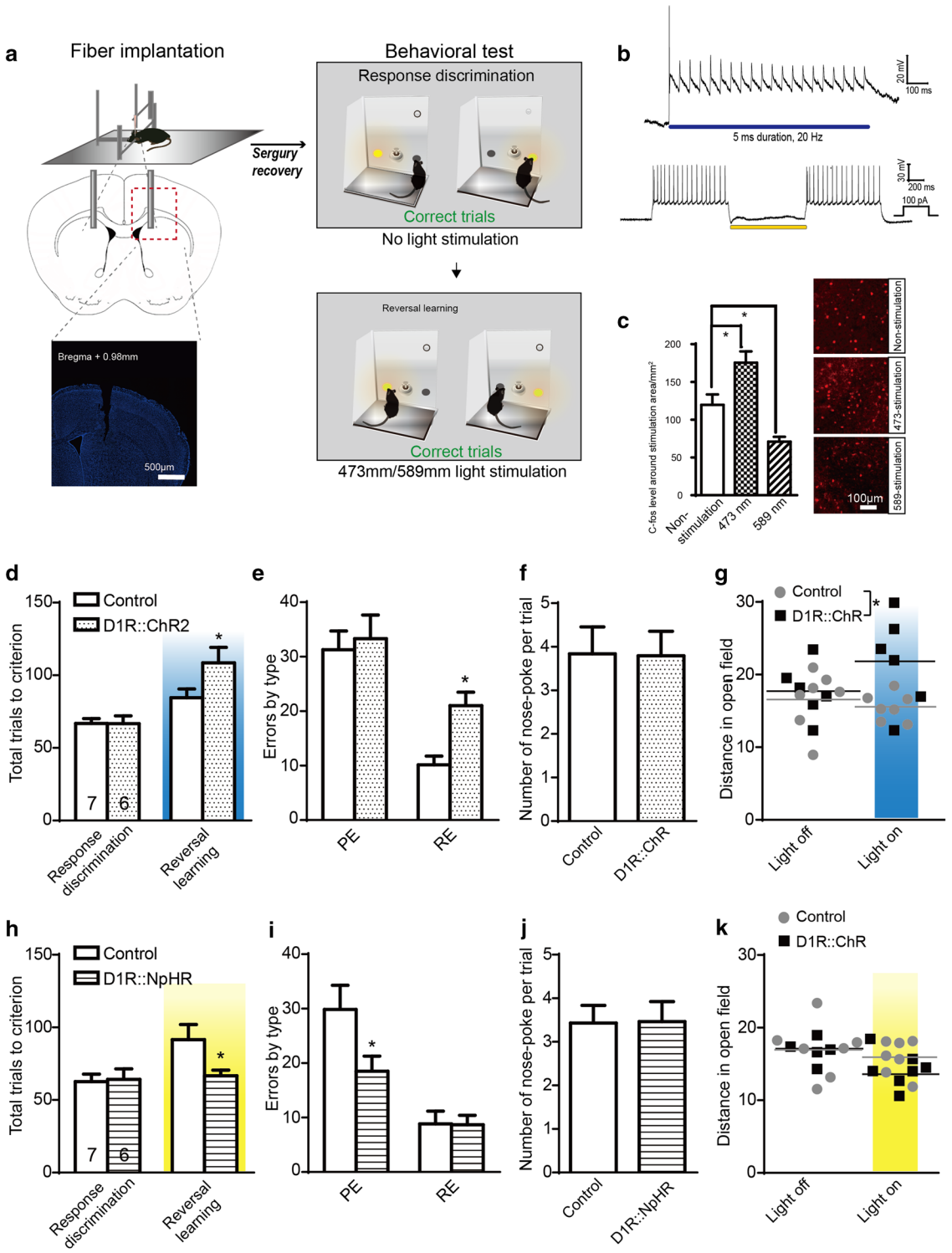
The serial reversal learning processes were identical to reversal learning initially. After the habituation and response discrimination stages, the mice would go through a 3-day serial reversal stage. During these days, the correct direction would be daily changing (right–left–right or left–right–left), and the mice had to learn the reversed strategy each day. The criterion for learning completion was the same as the reversal learning before.

Open field test

White colored plastic boxes were used as the open field chambers. The dimension of the boxes was $40\times40\times30$ cm. Mice were individually placed into the centers of the chambers and allowed to explore freely for 10 min. The locomotion and exploratory behaviors of mice were recorded with the Xeye Aba 3.2 tracking system (Beijing Macroambitior S&T Development Co., Ltd, Beijing, China). The center area was defined as the 17×17-cm area in the center of the chamber. The traveling distance and the time spent in the center area were also recorded. The total traveling distance was used to evaluate locomotor activity.

Immunohistochemical analysis

The mice were anesthetized and intracardially perfused with 50 ml of 0.9% saline followed by 100 ml of 4% paraformaldehyde/phosphate buffer 90 min after the behavioral tests. Brains were removed and postfixed in 4% paraformaldehyde for 6 h and then immersed in 30% sucrose solution for 48 h successively. Coronal sections (40 μm thick) were cut on a cryostat, and slices were stored in phosphate buffered saline (PBS) and processed for c-Fos immunohistochemistry. The brain sections were incubated in blocking buffer that contained 0.5% Triton X-100 and 10% normal goat serum for 90 min. After blocking, the sections were incubated with the c-Fos (1:400; Abcam) primary antibody in blocking buffer at 4 °C for 10 h. After washing, the sections were incubated with the goat anti-rabbit AlexaFluor 546 secondary antibody (1:500; Invitrogen) for 2 h at room temperature. After extensive washing with PBS, the sections were mounted onto slides with a mounting medium (Vectashield) and visualized under a microscope. The c-Fos expressions were quantified using the ImageJ software. Two independent investigators who were blind to the mice group design performed cell counting. The scale in the photographs was set in ImageJ based on the physical dimensions of the photograph that were recorded with the Leica microscope system. The photographs were transformed from RGB color mode into 8-bit mode, and then a "threshold" of the optical density was set in the software based on the non-specific background staining. Only stains that were above the settled limit would be included in the quantification to be defined as c-Fos signals.



Statistical analyses

After behavioral testing, the mice were sacrificed. The brains were removed, fixed in a 4% formalin solution, frozen, sliced into 40-μm coronal sections, and mounted on slides. The viral expressions and optic fiber placements were verified. Data from the mice with proper viral expressions and optic fiber placements were included for the analyses. The data were analyzed using the GraphPad Prism 6.0 software. The results are expressed as the mean ± standard error of the mean (SEM). The data of "total trials to criterion" and "error types" in reversal learning task were analyzed using two-way

◂**Fig. 2** Surgery and behavioral test procedures are shown as **a**. Fibers were planted into the aDMS (AP, + 1.1 mm; ML, ± 1.4 mm; DV, −2.5 mm) (**a**, left panel). Two weeks later, mice were put into the operant chambers to conduct behavioral tests (**a**, right panel). Mice were trained to perform place–response discrimination during the initial learning day. Twenty-four hours later, mice were required to learn a reversal direction of the response rule, in which the mice had to nose poke in the hole that was opposite that which they learned the previous day. During the reversal learning period, a blue light (473 nm, 20 Hz, 5 ms stimulation duration per 5 s) or yellow light (589 nm, 2900 ms constant stimulation with 100 ms interval) was conducted in the aDMS. **b** In a D1R::ChR mouse, whole-cell slice recording showed blue light-evoked action potential in ChR2 cells in the aDMS and yellow light-suppressed action potential in NpHR cells. **c** D1R-MSNs expressing ChR2 showed a significant increase in c-Fos activation following 40 min of blue light stimulation, whereas a significant decrease in c-Fos activation occurred in D1R-MSNs expressing NpHR following 40 min of yellow light stimulation. Activating D1R-MSNs in the aDMS slowed down reversal learning, especially increasing REs. The total number of trials and errors is represented in **d, e**, respectively. Inhibiting D1R-MSNs in the aDMS promoted reversal learning, mainly reducing PEs. The total number of trials and errors is shown in **h, i**. Modulating D1R-MSNs in the aDMS had no effect on the nose pokes/trial during the reversal phase. The average number of nose pokes per trial is presented in **f, j**. Activating D1R-MSNs in the aDMS enhanced the moving distance in the open field, while inhibiting D1R-MSNs had no significant effect on locomotion. The moving distance during different stages is shown in **g, k**

ANOVAs, with treatment as the between-subject factor and phase/error type as the within-subject factor. Significant main effects were followed up with multiple comparisons using Bonferroni post-tests. The data of the number of nose pokes per trial, the distance in the open field, and the c-Fos$^+$ cell number were analyzed with a two-tailed *t*-test. The criterion for statistical significance was set at $p < 0.05$.

Results

Distinct roles of D1R- and D2R-MSNs in the aDMS in modulating reversal learning

We first clarified cell-type-specific functions in aDMS neurons in modulating cognitive flexibility. To manipulate optogenetically D1R-MSNs or D2R-MSNs in the aDMS specifically, we crossed D1R-Cre or A2R-Cre mice with Cre-dependent ChR Ai32 mice or Cre-dependent NpHR Ai39 mice (Fig. 1a). The slice images show that light-sensitive proteins can be expressed in striatal direct and indirect pathways, respectively (Fig. 1b). Fibers were implanted 500 μm above the main targeted areas to target D1R-MSNs or D2R-MSNs in the aDMS for optogenetic manipulation (Fig. 2a, left panel). The mice were put into a reversal learning task 2 weeks after the surgery (Fig. 2a, right panel). We used two separate sets of D1R::ChR mice to probe the active/inhibitory efficiency of light stimulation by electro-physiology and c-Fos immunostaining, respectively. A blue light evoked the action potential in ChR2 cells in the aDMS, while a yellow light-suppressed action potential in NpHR cells (Fig. 2b). D1R-MSNs expressing ChR2 showed a significant increase in c-Fos activation following 40 min of blue light stimulation, whereas a significant decrease in c-Fos activation occurred in D1R-MSNs expressing NpHR following 40 min of yellow light stimulation (Fig. 2c).

Two-way repeated measure ANOVA analyses revealed a significant Treatment × Phase interaction effect in D1R-MSNs activating manipulation ($F_{1,11} = 6.923$, $p = 0.0234$; Fig. 2d) and a marginal trend toward a significance in interaction effect in D1R-MSN inhibition ($F_{1,11} = 4.015$, $p = 0.0703$; Fig. 2h). Through the Bonferroni post hoc test, we found that mice with D1R-MSN activation in the aDMS showed difficulty in reversal performance ($p = 0.0363$), and inhibiting D1R-MSNs enhanced reversal learning ($p = 0.050$). Further error-type analyses exhibited that activating D1R-MSNs in the aDMS mainly increased REs ($p = 0.0412$; Fig. 2e), while inhibiting D1R-MSNs mainly reduced PEs ($p = 0.034$; Fig. 2i) during the reversal phase.

Similarly, we conducted reversal learning testing with D2R-MSN activation and inhibition (Fig. 3a). Activating D2R-MSNs in the aDMS showed no treatment effect ($F_{1,14} = 1.763$, $p = 0.2055$) or Treatment × Phase interaction effect ($F_{1,14} = 1.301$, $p = 0.2731$; Fig. 3b). However, further analyses of error type revealed a significant reduction in PEs ($p = 0.011$; Fig. 3c). In contrast, inhibiting D2R-MSNs showed no obvious treatment effect on reversal learning, with no difference in total trials to criterion ($F_{1,11} = 0.3151$, $p = 0.5858$; Fig. 3f) or in the number of PEs or REs ($F_{1,22} = 0.0066$, $p = 0.9359$; Fig. 3g).

To rule out the influence of the motivation to get the reward, we analyzed the nose poke/trial during the reversal phase. There was no difference observed between any of the groups, suggesting the promoted or decreased performance in reversal learning was not due to a change in learning motivation (All $p > 0.05$; D1R-MSN manipulations: Fig. 2f, j; D2R-MSN manipulations: Fig. 3d, h).

Because the striatum mainly influences locomotion, we tested basic activity in the open field while optogenetically modulating the D1R-/D2R-MSNs in the aDMS. Activating D1R-MSNs enhanced the moving distance in the open field ($p = 0.0276$; Fig. 2g), while inhibition of this type of neuron had no significant effect ($p > 0.05$; Fig. 2k). On the contrary, activating D2R-MSNs had no obvious effect on locomotion ($p > 0.05$; Fig. 3e), while inhibiting D2R-MSNs promoted moving distance ($p = 0.0352$; Fig. 3i). These data indicate that altered reversal learning abilities are not attributable to possible roles of the striatum in motor regulation.

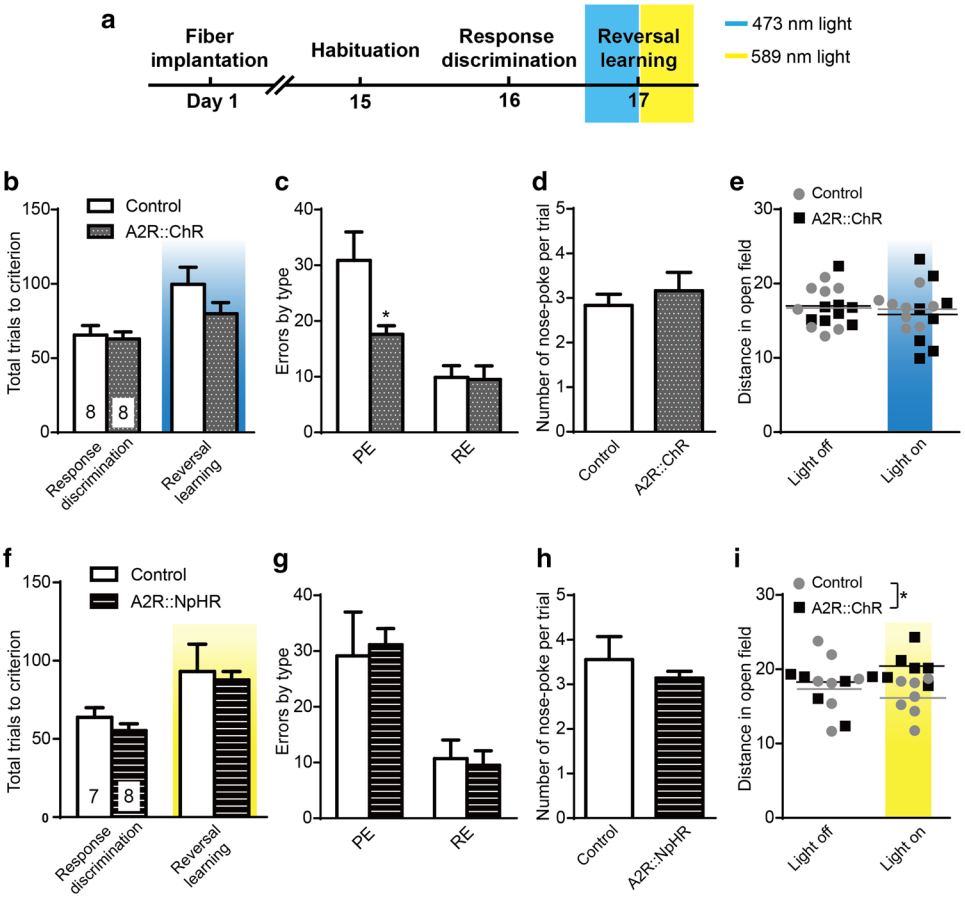


Fig. 3 The behavioral test procedure is shown as **a**. The mice were put into behavioral tests, as in previous studies. Activating D2R-MSNs in the aDMS slightly promoted reversal learning, mainly by decreasing PEs. Inhibiting D2R-MSNs in the aDMS had no obvious effect on reversal learning. The total number of trials and errors is represented in **b, c, f, g** respectively. Modulating D2R-MSNs in the aDMS had no effect on the nose pokes/trial during the reversal phase. The average number of nose pokes per trial is presented in **d, h**. Activating D2R-MSNs had no obvious effect on locomotion, while inhibiting D2R-MSNs increased the moving distance. The moving distance during different stages is shown in **e, i**

D1R-MSNs manipulation induced stage-dependent behavioral modulation during reversal learning

We unexpectedly found that activating and inhibiting aDMS D1R-MSNs, respectively, had major effects on the late (reducing REs) and early (enhancing PEs) stages of reversal learning in previous experiments (Fig. 2c, g). To confirm further the function of D1R-MSNs during different stages of reversal performance, we performed bi-direct optogenetic modulation in D1R-MSNs of the same subjects in a series of reversal learning tests. We first crossed the Cre-mediated ChR Ai32 mice with Cre-mediated NpHR Ai39 mice to create offspring that contained both the Cre-mediated ChR and NpHR proteins. We then used these offspring to cross with D1R-Cre mice to get mice in which D1R-MSNs could be both inhibited and excited in one subject (Fig. 4a).

Two weeks after the fiber implantation surgery, the mice were put into the serial reversal learning tests. There were no neuronal manipulations on the first day of reversal, the D1R-MSNs in the aDMS would be inhibited on the second day, and the D1R-MSNs would be excited in the same group of mice on the third day (Fig. 4b). Consistent with previous results, inhibiting D1R-MSNs in the aDMS promoted reversal learning, whereas activating D1R-MSNs slowed reversal learning. Two-way repeated measure ANOVA revealed a significant Treatment × Phase interaction effect ($F_{3,56} = 5.32$, $p = 0.0027$; Fig. 4c). A Bonferroni post hoc test showed that D1R-MSNs inhibited mice, who performed fewer trials to reach the criterion ($p = 0.0453$), while D1R-MSNs-excited mice performed more trials ($p = 0.0178$). Analyzing the error types revealed that inhibiting D1R-MSNs significantly reduced PEs ($p = 0.0008$; Fig. 4d), while exciting D1R-MSNs increased REs ($p = 0.0008$; Fig. 4e). We supposed that the stimulation order might have minimal effect on behaviors. First, according to the results from serial reversal learning, we observed similar behavioral changes comparable to the previous reversal learning experiments (Fig. 2). Second, because optogenetic stimulation is a kind of acute modulation in precise time, there would be no prolonged impact on the targeted neurons (Deisseroth 2015).

No effect of manipulating aDMS neurons in the acquisition or retention of discrimination learning

To clarify whether specific activation and inhibition in D1R- or D2R-MSNs of the aDMS could affect basic learning ability and memory, we used four separate sets of mice

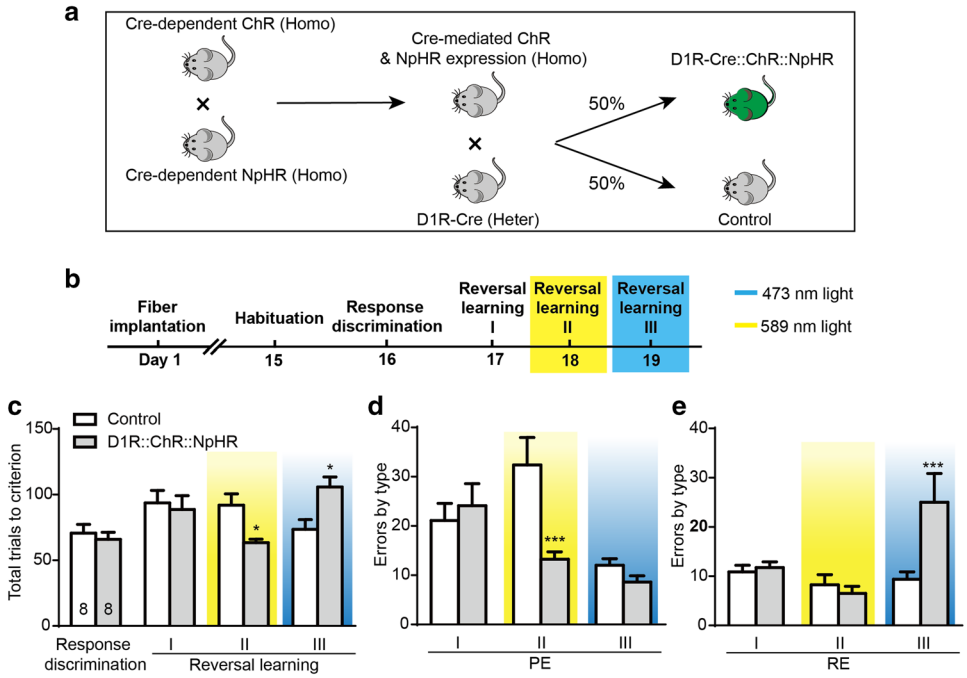


Fig. 4 By crossing the Cre-mediated ChR Ai32 mice with the Cre-mediated NpHR Ai39 mice, we created offspring that contained both the Cre-mediated ChR and NpHR proteins (illustrated with green); **a** then, using these offspring to cross with D1R-Cre mice, we got mice in which D1R-MSNs could be both inhibited and excited in one subject. The behavioral test procedure is shown in **b**. Two weeks after the fiber implantation surgery, the mice were put into the serial reversal learning tests. The D1R-MSNs in the aDMS were activated or inhibited on the second and third days of serial reversal learning. The total number of trials and errors is represented in **c**–**e**. Activating D1R-MSNs promoted reversal learning, and it especially affected the REs, while inhibiting D1R-MSNs reduced reversal learning, mainly affecting PEs

to conduct another battery of behavioral tests, during which the D1R/D2R-MSNs in the aDMS were constantly stimulated throughout the initial learning retention phase and reversal learning tasks (Fig. 5a). Mice showed no difference in the response discrimination period or retention test (All $p > 0.05$) in D1R-MSN/D2R-MSN activation or inhibition (Fig. 5b–e). The effects of optogenetic stimulation were only observed in reversal learning (D1R::ChR: $p = 0.0074$; D1R::NpHR: $p = 0.020$; D2R::ChR: $p = 0.023$; Fig. 5b–d), except for there being no change in D2R-MSN inhibition ($p = 0.334$; Fig. 5e). These findings indicate that manipulating any type of MSN in the aDMS does not affect the acquisition or retention of discrimination learning.

Alterations in neuronal activities in the MD thalamus associated with altered reversal performance

To elucidate whether the MD thalamus participates in striatum-controlled flexible behavior, we perfused the mice and analyzed the c-Fos expression in the MD thalamus (Fig. 6a) following the reversal learning task above. A Student *t*-test analysis was used, and we observed c-Fos changes that related positively to behavioral performance. While mice showed an impaired ability in reversal performance, the c-Fos level was decreased in the MD thalamus (D1R::ChR: $t_7 = 3.941$, $p = 0.0056$; Fig. 6b). Conversely, when mice showed an enhanced ability in reversal learning, the c-Fos expression in the MD thalamus increased (D1R::NpHR: $t_7 = 3.478$, $p = 0.0103$; A2R::ChR: $t_6 = 2.753$, $p = 0.0331$; Fig. 6c, d). No effect was found on the c-Fos expression when no change was shown in a behavioral test (A2R::NpHR: $t_5 = 0.0641$, $p = 0.9541$; Fig. 6e).

In fact, activation in the striatal direct and indirect pathways typically activates and suppresses the neurons in the MD thalamus, respectively (Mitchell 2015; Grillner and Robertson 2016). Our findings seem inconsistent with this. To dissect the phenotypes of neuronal activity in the MD thalamus resulting from behavioral performance or simple light stimulation in MSNs, we tested the c-Fos levels after light stimulation in D1R-MSNs and D2R-MSNs without a reversal learning task using a separate set of mice. The results presented that activating D1R-MSNs in the aDMS enhanced neuronal activity in the MD thalamus with an increased c-Fos expression ($t_6 = 2.593$, $p = 0.0410$; Fig. 7a), while activating D2R-MSNs in the aDMS decreased the c-Fos expression in the MD thalamus ($t_6 = 3.956$, $p = 0.0075$; Fig. 7b). In this experiment, the

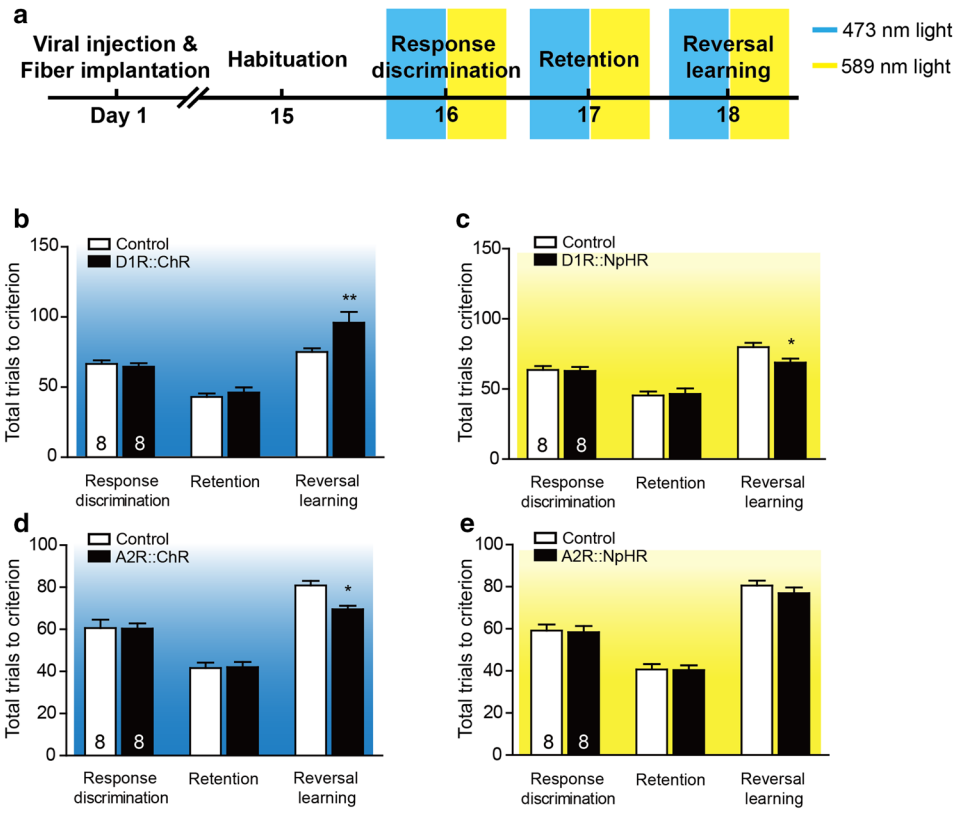


Fig. 5 The behavioral test procedure is shown in **a**. Two weeks after fiber implantation surgery, mice were put into behavior tests. The mice were trained to conduct place–response discrimination, resembling previous studies. One day later, they were put into the exact same learned task to consolidate the initial learning. Then, 24 h later, the mice were required to do the reverse of the learning task. Throughout the whole behavior test, a blue or yellow light was given to modulate targeted neurons (the same stimulation parameters as previous). The data in different manipulations are shown in **b**–**e**, respectively. Activating or inhibiting D1R/D2R-MSNs in the aDMS did not interfere with the initial learning or retention phase, while the influence on reversal learning was consistent with our former findings

mice went through a 3-day procedure. The protocol on each day was the same as that on the habituation day of the reversal learning task, when the mice received exactly the same condition information with the sucrose reward via random release, and the light in the nose-poke hole was illuminated randomly on the second and third days. Especially, light stimulation was conducted on the third day. For a comparison, we conducted this control task for 40 min, which was matched with the reversal learning test. Taken together, these data revealed that the previous c-Fos changes in the MD thalamus after reversal learning were behavioral based, and they might be due to the modulation inside the DMS or the projections between the DMS and MD thalamus.

To identify further whether there was a "hyper-direct" pathway between the aDMS and the MD thalamus, we traced the neuronal projections of aDMS D1R-MSNs by microinjecting Cre-inducible mCherry AAVs into D1R-Cre mice (Fig. 8a). A histological analysis showed there were sparse projections to the MD thalamus besides the main projections to the GPi and SNR. Then, we directly manipulated the aDMS–MD circuit to demonstrate whether these projections participate in reversal learning (Fig. 8b). There was no effect on either total criterion in reversal learning ($F_{1,12}$ = 1.432, $p = 0.2546$; Fig. 8d) or locomotion in the open field ($p > 0.05$, Fig. 8c), despite a decrease in the c-Fos level in the MD thalamus ($t_{10} = 2.260$, $p = 0.0473$; Fig. 8e).

Discussion

The present study first revealed that the aDMS engaged in the regulation of cognitive flexibility in a cell-type-specific manner. The activation of D1R-MSNs in the aDMS impaired reversal learning; on the contrary, the activation of D2R-MSNs in this brain area facilitated the strategy. Compared to D2R-MSNs, D1R-MSNs played a more important role in this cognitive regulation. Accordingly, the neuronal activity in the MD thalamus, a downstream brain region of the striatum in the mPFC–striatum–thalamus circuit, was down-regulated or up-regulated, which was closely related to the disruption or facilitation of reversal performance.

Reversal learning includes striatal dopaminergic modulation

Dopamine (DA) neurons in the ventral tegmental area (VTA) and the substantia nigra (SNc) encode errors in reward prediction (Schultz 2001). The firing rate of a DA neuron is increased when a reward is expected, and the firing is decreased when an expected reward is omitted, which are involved in the strategy reversing processes. In other words, when the DA neuron activations correspond more to their predictions than to the reward, the errors in reward prediction have a greater influence on the DA neurons (Hollerman et al. 1998). This might underlie strategy switching, during

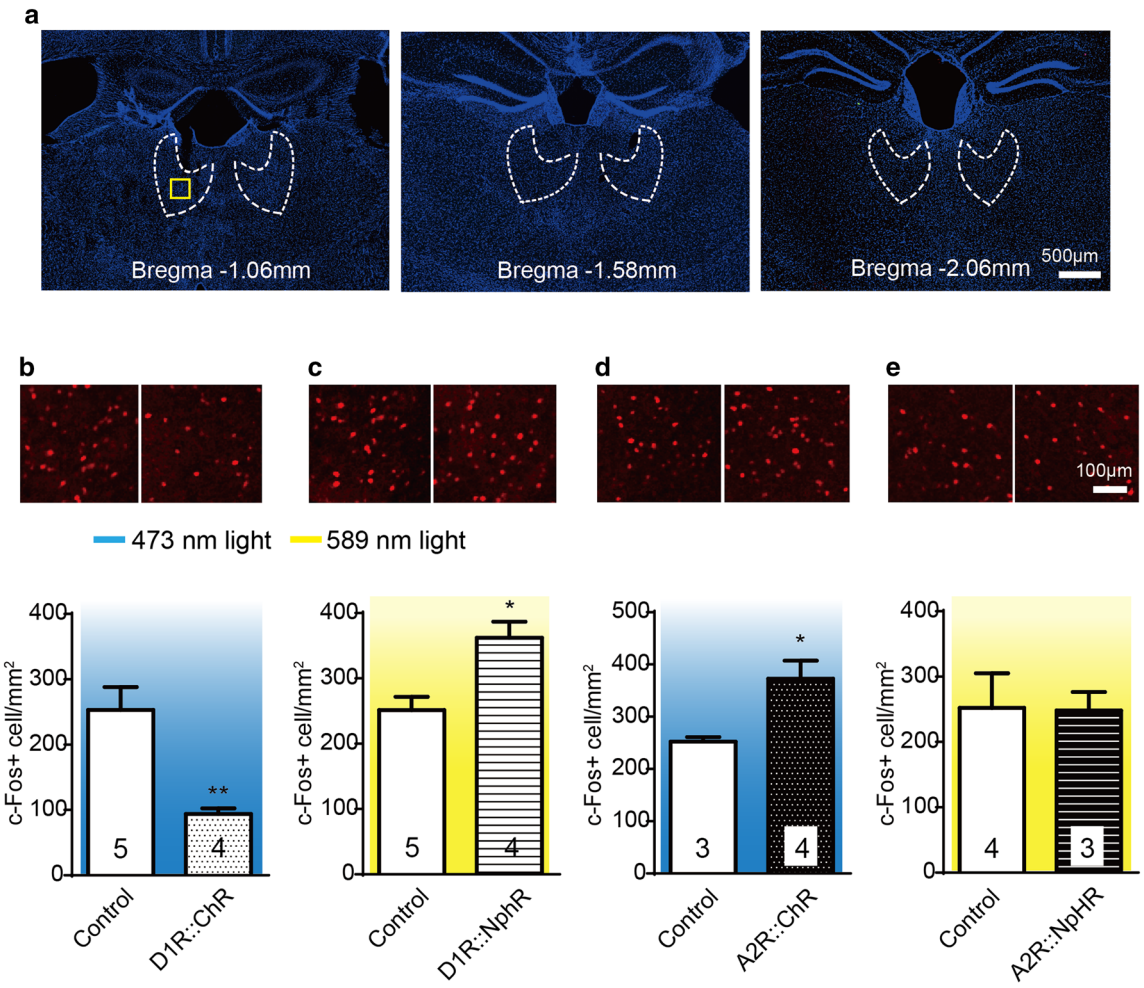


Fig. 6 The range for the c-Fos analysis in the MD thalamus (**a**; white dashed boxes: the areas analyzed in each section; yellow solid boxes: the positions of the magnified images shown in Fig. 5b–e). The c-Fos changes were related to the behavioral performance. By activating D1R-MSNs, mice showed poorer ability in reversal performance, and the c-Fos level was decreased in MD. Conversely, by inhibiting D1R-MSNs or activating D2R-MSNs, mice showed a promoted ability in reversal learning, and the c-Fos expression in the MD thalamus increased. Inhibiting D2R-MSNs had no effect on the c-Fos expression; meanwhile, no change was shown in the behavioral test. The example illustration of the c-Fos expression and the number calculation are shown in **b–e**

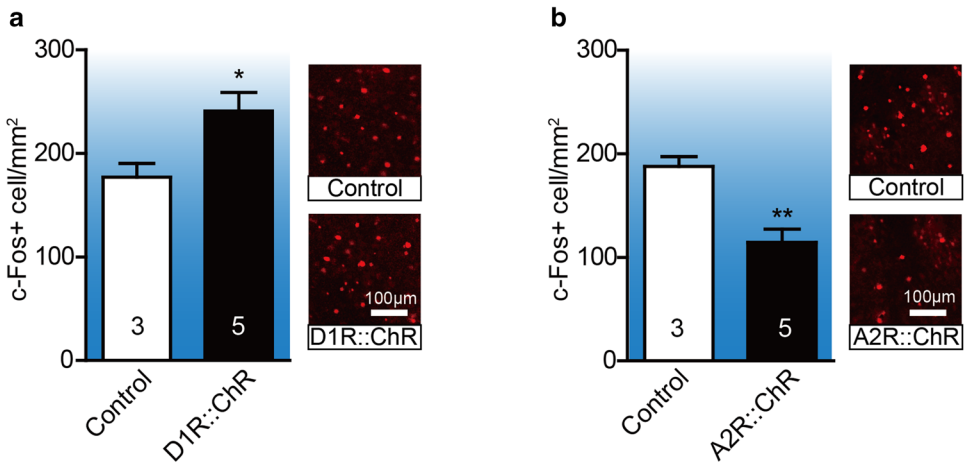


Fig. 7 By activating D1R-MSNs in the aDMS by optogenetic stimulation, the c-Fos expression increased in the MD thalamus. While activating D2R-MSNs in the aDMS by optogenetic stimulation, the c-Fos expression decreased in the MD thalamus. The c-Fos illustration and number calculation are represented in **a, b** respectively

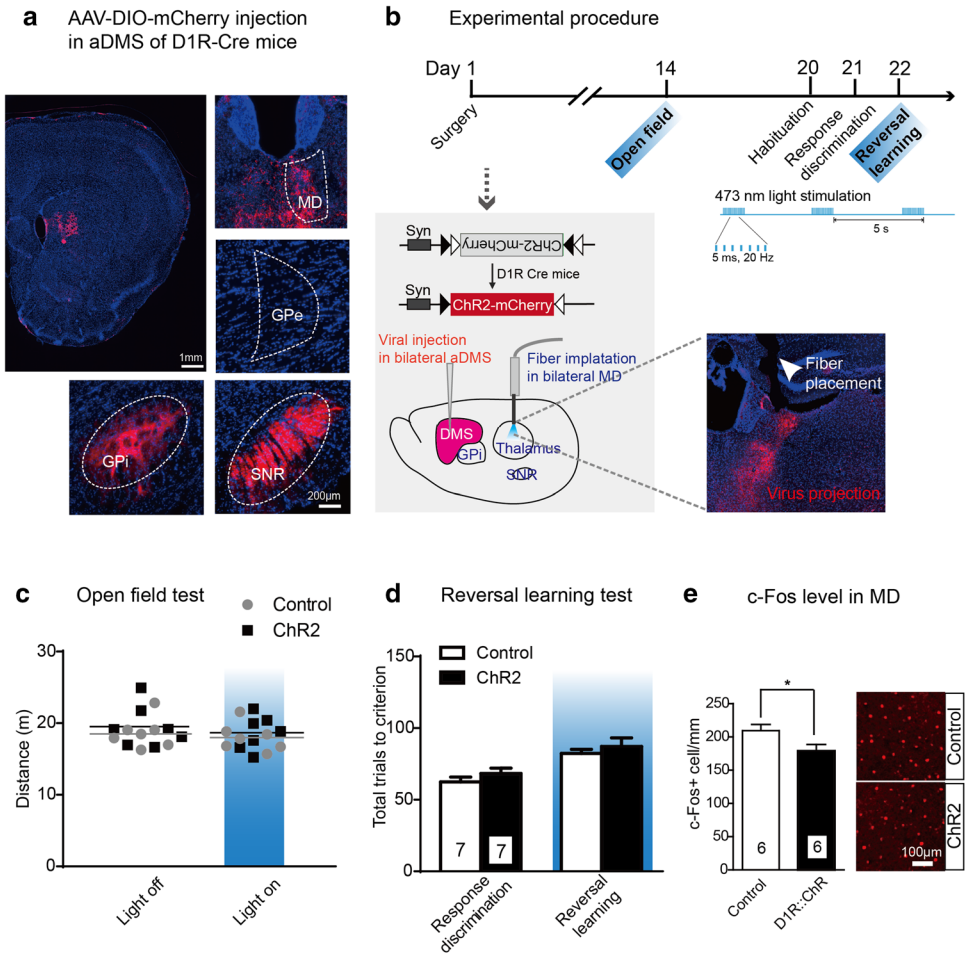


Fig. 8 The neuronal projections of aDMS D1R-MSNs were marked by expressing the Cre-inducible mCherry virus (AAV-DIO-mCherry) in D1R-Cre mice. The local expression of the virus was mainly in the aDMS (left panel, red, scale bar 1 mm; **a**) and D1R-MSNs in the aDMS mainly projected to the GPi and SNR. There were also sparse projections to the MD thalamus. No projection was observed in the GPe (right panel, red, and scale bar 200 μm; **a**) and the behavioral test procedure is shown in **b**. Viruses were injected into the aDMS while fibers were planted into the MD thalamus (middle, lower panel; **b**). Two weeks after the surgery, mice were put into behavioral tests, which was similar to previous tests. The circuits would be selectively modulated during reversal learning (upper panel; **b**). Activating the D1R-MSNs in the aDMS–MD pathway had little effect on locomotion or reversal learning. The moving distance in the open field and the errors in reversal learning are shown in **c, d**, respectively. Directly exciting D1R-MSNs in the aDMS–MD pathway slightly reduced the c-Fos expression in MD ($n=6$ in each group; 3–5 slices were analyzed for each mouse). The c-Fos expression and number calculation are illustrated in **e**

which time individuals gradually notice the environmental changes and learn to switch from the acquired but outdated strategy to an updated one. Previous studies have not only demonstrated that the VTA/SNc-DMS terminals strongly respond to reward prediction-related signals (Parker et al. 2016), but they also revealed that DA projections from the VTA and SNc play significant roles in striatum functions (Heien and Wightman 2006). This suggests that midbrain-DMS DA modulation might be highly engaged in the cognitive flexibility process. Indeed, clinical studies have reported that L-DOPA-medicated Parkinson's disease patients exhibited impaired reversal learning with intact initial acquisition (Cools et al. 2001, 2007a), indicating that a DA overdose in the striatum resulting from L-DOPA medication is specifically implied in cognitive inflexibility (Cools et al. 2003). This implication is also supported by animal studies. For example, reversal learning deficits were observed following endogenous DA administration in mice (Robinson et al. 2005). With extended drug self-administration, there was elevated DA efflux in the dorsal striatum, leading to excessive stamping of associations between stimuli and responses (Ito et al. 2002). These findings indicate that altered activity in the striatal neurons in relation to an abnormal DA level may contribute to deficits in cognitive flexibility. In other words, while prediction errors are coded by dopaminergic neurons in the midbrain, the striatum, which receives

dopaminergic projections, appears critically involved in the reversal process.

Bidirectional modulation of the striatal direct and indirect pathways to reversal learning

Among the 95% GABA MSNs in the striatum, the striatonigral MSNs projecting to the GPi and SNR express D1Rs, whereas striatopallidal MSNs projecting to the GPe express D2Rs (Gerfen et al. 1990). The use of pharmacological tools that target D1Rs and D2Rs has helped to reveal the relative contribution of the direct and indirect pathways to cognitive flexibility regulation. Some studies identified a role for D1R signaling in cognitive flexibility (McLean et al. 2009; Bestmann et al. 2015), while others showed the importance of D2R signaling (Cools et al. 2007b; Haluk and Floresco 2009). However, there are inconsistent reports that D1R or D2R inhibition is insufficient to affect cognitive flexibility (Calaminus and Hauber 2007; Bestmann et al. 2015). Besides different models of drug action on receptors, the complex expression pattern of D2R present in both pre- and postsynaptic compartments may partially complicate the interpretation of these experimental results (Lindgren et al. 2003; Morita et al. 2016). Using cell type-specific manipulation, in the present study, we mainly highlight an important implication for understanding how D1R- and D2R-expressing neurons in the aDMS function in rule update. D1R- and D2R-MSNs performed opposing actions, with more important roles of D1R-MSNs in cognitive regulation (Figs. 2, 3). By analyzing the error type, we further revealed that modulating the D1R-MSNs significantly affected the early stage of reversal learning, and the effect was maintained throughout the whole phase, but the D2R-MSNs only affected the early part of reversal learning. The engagement of D1R-MSNs in the aDMS during behavioral reverse seems based on their status (Charntikov et al. 2017). In the early stage of reversal learning, D1R-MSNs are hyper-activated to cope with the changing situation and the different response rules (Nakanishi et al. 2014). During the learning process, the activities of D1R-MSNs gradually decrease and become stable to maintain the new strategy. Correspondingly, to switch the strategy successfully, the activities of D2R-MSNs must be enhanced in the early stage of reversal learning to keep balance with the abnormally increased D1R-MSN activities (Cheng et al. 2017) (Fig. 4). This hypothesis is supported by many previous studies that showed how D1R and D2R worked together through competing but also balanced ways on reward-based cognitive functions (Agnoli et al. 2013; Soares-Cunha et al. 2016). Nevertheless, our current study lacks direct evidence that D1R- and D2R-MSNs exact interaction in reversal learning, necessitating further investigation.

The DA receptor system is importantly involved in motor regulation (Kravitz et al. 2012). However, the present effect on reversal learning is unlikely driven by altered motor actions because flexibility and motor actions were not affected in parallel in MSN manipulations (Figs. 2, 3). For example, the inhibition of D1R-MSNs impaired reversal learning, but locomotor activity was not significantly changed. Additionally, our data for the initial acquisition and retention in the first task indicate that basic mechanisms of feedback-based learning were intact following neuronal activation or inhibition in the aDMS (Fig. 5). Taken together, an altered reversal performance induced by aDMS modulation reflects changes in a complex feedback system, rather than influenced non-specific motor or basic learning ability.

Striatum-involved neural circuits are important in reversal learning

The striatum is the largest component of the basal ganglia circuitry and the primary location at which information is passed into those circuits from the cortex and thalamus. Anatomically, the head of the caudate is an area heavily connected with the dorsolateral regions of the frontal lobe via terminal cortical afferents in humans (Alexander et al. 1986), which is a crucial area for regulating cognitive function associated with behavioral flexibility (Daum et al. 1991; Fellows and Farah 2003). Abnormalities in the neuronal activation of the mPFC that project to the aDMS in rodents (Voorn et al. 2004) have been implicated in altered reversal learning performances (Graybeal et al. 2011). These findings imply that the aDMS area, located in the mPFC–aDMS–thalamus circuit, plays a potential role in cognitive regulation. In other words, the aDMS projection neurons, following DA signal input from the midbrain, integrate the information from the cortex and finally regulate behavioral strategies to adapt environmental changes through direct and indirect pathways (Surmeier et al. 2007).

In this neural circuit, the MD thalamus is an important relay nucleus. Neurons of the MD thalamus would be basically activated following the activation of D1R-MSNs in the striatum by the direct pathway, while its neuronal activity would involve the suppressed activation of striatal D2R-MSNs through the indirect pathway (Mitchell 2015). Consistent results were obtained in our study (Fig. 7). Surprisingly, we revealed some unexpected findings about the relationship between cell-type-specific manipulation in the aDMS and neuronal activity in the MD thalamus during the reversal task. The mice showing the impairment in reversal learning after D1R-MSN activation exhibited a significant decrease in the activity of the MD thalamic neurons, and opposite results were found when activating D2R-MSNs (Fig. 6). Seemingly paradoxical, the present observations are consistent with a previous report in which cognitive flexibility was impaired when MD thalamic neurons were damaged (Parnaudeau et al. 2015). Nevertheless, the alterations

in behavior and MD neuronal activity did not result from the modulation of "super direct" projections from the aDMS to the MD, though this pathway anatomically exists (Fig. 8). Viewed together, the present results indicate that network modulation from inhibitory interneurons inside the DMS may play a significant role of shaping the activity of neighboring striatal ensembles encoding alternative behaviors (Gage et al. 2010; Chuhma et al. 2011). The information modulation and integration by interneurons may mainly occur in the striatum rather than the globus pallidus, as there is a vast gap in neuron amounts between the two brain regions (111 million vs. 160,000) (Fox and Rafols 1976; Lange et al. 1976). This interpretation still needs to be demonstrated by further studies.

Conclusions

D1R- and D2R-MSN subtypes in the aDMS and their parallel pathways exert opposing actions on reversal behaviors, with more important and complex roles of D1R-MSNs involved. Mental disorders with a cognitive flexible problem may underlie the functional imbalance in the aDMS' two types of neurons and their projections. These cases suggest the possibility of stimulation in the aDMS-involved neural network for relieving rigid mental acts or behaviors.

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Author contributions XYW, YHQ and ZHD performed experiments; XYW analyzed data and wrote the manuscript; JL and NS designed experiments; FS and JJZ managed animal and designed animal model; JL and XYW finished the paper.

Compliance with ethical standards

Ethical approval Research involves animal participants. All procedures were approved by Institutional Review Board of the Institute of Psychology, Chinese Academy of Sciences, and were in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Informed consent All the co-authors are informed and in agreement on this submission. The authors declare that there is no conflict of interests to this work.

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