Received: 30 May 2023 | Revised: 13 July 2023 | Accepted: 23 July 2023

DOI: 10.1002/npr2.12369

NEUROPSYCHOPHARMACOLOGY REPORTS

WILEY

ORIGINAL ARTICLE

Inhibition of the dorsomedial striatal direct pathway is essential for the execution of action sequences

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Funding information
Japan Agency for Medical Research and Development, Grant/Award Number: JP19dm0207069; Japan Science and Technology Agency, Grant/Award Number: PRESTO/JPMJPR22S4 and SPRING/JPMJSP2123

Abstract

Contrary to the previous notion that the dorsomedial striatum (DMS) is crucial for acquiring new learning, accumulated evidence has suggested that the DMS also plays a role in the execution of already learned action sequences. Here, we examined how the direct and indirect pathways in the DMS regulate action sequences using a task that requires animals to press a lever consecutively. Cell-type-specific bulk Ca^{2+} recording revealed that the direct pathway was inhibited at the time of sequence execution. The sequence-related response was blunted in trials where the sequential behaviors were disrupted. Optogenetic activation at the sequence start caused distraction of action sequences without affecting motor function or memory of the task structure. By contrast with the direct pathway, the indirect pathway was slightly activated at the start of the sequence, but the optogenetic suppression of such sequence-related signaling did not impact the behaviors. These results suggest that the inhibition of the DMS direct pathway promotes sequence execution potentially by suppressing the formation of a new association.

KEYWORDS
action execution, direct pathway, dorsomedial striatum, indirect pathway, sequential behavior

1 | INTRODUCTION

Our daily life is flooded with massive repertoires of learned action sequences such as typing on a keyboard, playing piano, playing sports, and performing dance. We learn single units of action and concatenate them into a sequence to execute the learned sequence and attain the goal.[1,2] Patients with motor deficits, including those with Parkinson's or Huntington's disease, struggle to execute action sequences, leading to a failure of goal-oriented behaviors.[3–5] Previous studies have repeatedly demonstrated that the striatum plays a key role in the execution of goal-oriented behaviors.[6–14] However, the striatum can be classified into several functional subregions: dorsomedial; dorsolateral; ventromedial; ventrolateral striatum[6,8,9,11,12,14–16] and consists of two major molecularly distinct subpopulations: dopamine type-1 receptors expressing and dopamine type-2 receptors expressing medium spiny neurons (D1-, D2-MSNs),[17–24] which are proposed to regulate different functions.[25–30] Therefore, distinguishing such regional and molecular segregations in the striatum is crucial for further understanding the neural basis of sequence execution.

The dorsolateral part of the striatum (DLS) has been reported to be involved in the execution of learned action sequences, while the dorsomedial part of the striatum (DMS) has been believed to be important for the acquisition of the action-outcome contingency to

learn a proper action to obtain the goal. DLS neurons are more active after animals learn goal-oriented behavior,[14,31] and inactivation of DLS neurons impairs learned actions or habit formation.[16] In contrast, DMS neurons are more active when the animals are learning the action-outcome relationships, and the activity becomes less active after acquiring the goal-oriented behaviors.[14,31] Inactivation of DMS neurons impairs the acquisition of new action-outcome contingency[16,32] or behavioral flexibility.[33–36]

However, recent studies have suggested that the DMS may also play an important role in the execution of acquired sequential behavior.[2,12,37] Of note, Vandaele et al.[12] discovered two populations in the DMS that were inhibited or activated while animals performed action sequences. Moreover, these sequence-related activities were weakened in trials where animals did not complete the learned action sequence. To reveal the functions of the DMS on action execution, it is crucial to segregate the two major striatal populations: D1-MSNs, which send signals to the substantia nigra reticulata (SNr) and the ventral tegmental area directly, and D2-MSNs, project to the SNr via the external segment of the globus pallidus external (GPe).[17–24]

Given the evidence that the DMS is involved in the execution of behavioral sequences, we sought to characterize the activities of DMS direct and indirect pathways in a task requiring mice to perform lever press sequences to obtain a food reward. Using transgenic mice, we recorded the bulk Ca^{2+} activity in the direct and indirect pathways and observed that the direct pathway neurons were inhibited during lever pressing and that the inhibition was blunted in a trial where mice suspended lever pressing temporarily and impeded the execution of sequential lever pressing. The indirect pathway was slightly activated at the sequence start, but the sequence-related signaling was not correlated with the efficiency of sequences. Moreover, optogenetic activation of the direct pathway promoted sequence suspension, whereas optogenetic suppression of the indirect pathway did not impact the behaviors. These results show that the direct pathway in the DMS was inhibited at sequence execution, and such inhibitory signaling was essential for executing the sequential behavior.

2 | METHODS

2.1 | Animals

All animal procedures were conducted following the National Institutes of Health Guide for the care and use of laboratory animals and approved by the Animal Research Committee of Keio University. Experiments were conducted using 3- to 12-month-old male mice. All mice were maintained under a 12:12 h light:dark cycle (lights on at 8 a.m.), and the behavioral experiments were conducted during the light phase. D1-YC mice (Pde10a2-tTA::tetO-YCnano50; Adora2a-Cre triple-transgenic mice) were obtained by crossing Pde10a2-tTA mice,[38] tetO-YCnano50 mice,[39] and Adora2a-Cre mice.[40] D1-ChR2 mice [Pde10a2-tTA::tetO-ChR2(C128S)-EYFP and Adora2a-Cre triple-transgenic mice] were obtained by crossing Pde10a2-tTA mice and tetO-ChR2 mice[41] and Adora2a-Cre mice. D2-YC mice (Drd2-tTA::tetO-YCnano50 double-transgenic mice) were obtained by crossing Drd2-tTA mice[6] and tetO-YCnano50 mice. D2-ArchT mice (Drd2-tTA::tetO-ArchT-EGFP double-transgenic mice) were obtained by crossing Drd2-tTA mice and tetO-ArchT-EGFP mice.[42] The genetic background of all transgenic mice was mixed C57BL6 and 129SvEvTac. Genotyping for tetO-YCnano50 and tetO-ArchT-EGFP was previously described.[8,39,41,42]

2.2 | Stereotaxic surgery

Surgeries were performed using a stereotaxic system (SM-6M-HT; Narishige, Amityville, NY, USA). Mice were anesthetized with ketamine and xylazine (100 and 10 mg/kg, respectively, administered intraperitoneally).

For optical recordings at the DMS, D1-YC and D2-YC mice were unilaterally (right side or left side) implanted with an optic fiber cannula (CFMC14L05, 400 μm core diameter, 0.39 NA; Thorlabs, Newton, NJ, USA) into the DMS [+1.10 mm anteroposterior (AP), 1.25 mm mediolateral (ML) from bregma, 1.85 mm dorsoventral (DV) from the brain surface] according to the atlas of Paxinos and Franklin. The insertion side was randomly assigned. For optogenetic manipulations, D1-ChR2 and D2-ArchT mice were bilaterally implanted with a 200-μm core diameter optical fiber (0.39 NA; Thorlabs, Newton, NJ, USA) into the DMS at a 27° angle across from the median line (+0.98 mm AP, ±2 mm ML, 2.2 mm DV).

2.3 | Fixed ratio (FR) operant task

Mice were housed individually under conditions of food restriction. Their body weights were maintained at 85% of their initial body weight. Behavioral training and tests were performed under constant darkness in an aluminum operant chamber (21.6 × 17.6 × 14.0 cm; Med Associates, Fairfax, VT, USA). The apparatus was controlled by a computer program written in the MED-PC language (Med Associates). The mice were required to perform a fixed number of actions (lever press, LP, Med Associates, Fairfax, VT, USA) to attain a reward. A food magazine between two retractable levers (Med Associates, Fairfax, VT, USA) was located on the floor of the chamber. The lever on the left side was designated "active" (triggering delivery of a food reward), and the lever on the right side was "inactive" (no relation to food reward). Each trial began with the presentation of two levers. Presses on the lever on the left of the food magazine (reinforced side) were counted, and a reward pellet (20 mg each, Dustless Precision Pellets; Bio-serv, Flemington, NJ, USA) was dispensed to the magazine immediately after the required number of presses was made. The levers were retracted at the same time as the reward delivery. After food delivery, a 30-s intertrial interval (ITI) was added, during which levers were retracted, followed by the automatic starting of the next trial. The ITI allows time for mice to consume the food pellet.

The training started with the FR1 schedule, in which the mice obtained one food reward after each active LP. The mice were trained for 10–14 consecutive sessions on the FR1 schedule. After the FR1 schedule, the mice that completed more than 50 trials per session proceeded to the FR5 schedule. The FR5 task required 5 lever presses to gain a reward. In each training session, the levers were presented for 60 s, and one session lasted for 60 min or until the mice received 100 food rewards. If the mice pressed the required number of active LPs during the lever presentation, the levers were retracted, and one food pellet was delivered ("completed trial"). After food delivery, there was a 30-s ITI, during which levers were retracted, followed by automatic commencement of the subsequent trial. If the mouse did not press the active lever during lever presentation, the levers were retracted, and the trial ended ("omitted trial"). Following the ITI, a new trial was begun.

We collected data from mice that experienced the FR5 task seven times. In the FR5 task, the mice occasionally poked their nose into the magazine before making the required number of lever presses,[43] so that the lever press sequence was distracted by checking the food magazine. After checking the magazine, the mice went back to the active lever zone and re-engaged to press a lever. The active lever zone and food magazine were 6 cm apart. The timing of entry into the magazine was defined as the time point when the distance of the mouse's head to the center of the magazine became less than 2.5 cm. By contrast with this distracted sequence, we called continuous lever pressing without checking a magazine a focused sequence. Moreover, we determined the nonmagazine area, which was a circle 2 cm in diameter and 10 cm apart from the active lever toward the opposite wall. We analyzed the frequency with which mice entered this area and examined the locomotor activity which was irrelevant to lever pressing.

To track the moment-to-moment position of the mice, an infrared video camera (ELP 2 Megapixel Web Camera, OV2710; Ailipu Technology Co., Ltd, Shenzhen, China) was attached to the ceiling of the enclosure. Reflective tape was attached to the optical fiber protector (1.2 × 1.4 cm) on the head of the mice. The tapes were recorded at a sampling rate of 20 Hz. The mice's position in each frame was computed offline by a custom-made MATLAB code.

On average, it took 25 days for surgery, recovery, and the entire behavioral procedure, including training. TTL signals were generated at the timings of the lever extension and lever press and digitized by a data acquisition module (cDAQ-9178; National Instruments, Austin, TX, USA). The TTL signals were simultaneously recorded at a sampling frequency of 1000 Hz by a custom-made LabVIEW program (National Instruments, Austin, TX, USA).

2.4 | Fiber photometry

The method for fiber photometry has been described previously.[8] An exciting light (435 nm; silver-LED; Prizmatix, Holon, Israel) was reflected off a dichroic mirror (DM455CFP; Olympus, Tokyo, Japan), focused with a 20× objective lens (NA 0.39; Olympus, Tokyo, Japan) and coupled into an optical fiber (M79L01, 400 μm core diameter, 0.39 NA; Thorlabs, Newton, NJ, USA) through a pinhole (400 μm diameter). The LED power was <100 μW at the fiber tip. Emitted cyan and yellow fluorescence from YCnano 50 was collected via an optical fiber cannula, divided by a dichroic mirror (DM515YFP; Olympus, Tokyo, Japan) into cyan (483/32 nm band-path filters; Semrock, Rochester, NY, USA) and yellow (542/27 nm) and detected by each photomultiplier tube (H10722-210; Hamamatsu Photonics, Shizuoka, Japan). The fluorescence signals and TTL signals from behavioral settings were digitized by a data acquisition module (cDAQ-9178; National Instruments, Austin, TX, USA) and simultaneously recorded using a custom-made LabVIEW program (National Instruments, Austin, TX, USA). Signals were collected at a sampling frequency of 1000 Hz.

2.5 | Optogenetic manipulation

For optogenetic activation, blue and yellow light (0.1 s duration, respectively) was used to open and close the step-function opsin ChR2(C128S).[44] Six D1-ChR2 mice were subjected to the FR5 task under optogenetic activation. In the control trials, yellow light was used instead of blue light in the same six D1-ChR2 mice. Five D2-ArchT mice were subjected to the FR5 task under optogenetic suppression. For optogenetic inhibition, a 0.2 s duration of yellow (inhibition) light was used in D2-ArchT mice. In control sessions, blue light was used in the same five D2-ArchT mice. Optogenetic manipulation was applied to the mice after they had completed 50 trials per FR5 session and experienced the FR5 task seven times. The manipulation (stimulation and control, counterbalanced) was conducted four times.

Optical fibers (NA 0.39; Thorlabs, Newton, NJ, USA) were inserted bilaterally through the guide cannulae. Yellow (575 nm) and blue (475 nm) light were generated by a Spectra 2-LCR-XA light engine (Lumencor, Beaverton, OR, USA). The yellow and blue light power intensities at the tip of the optical fiber were 3–4 and 2–3 mW, respectively. The TTL pulses generated by MED-PC (Med Associates, Fairfax, VT, USA) controlled the light.

2.6 | Immunohistochemistry

Following completion of each experiment, mice were deeply anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg), perfused intracardially with 4% paraformaldehyde phosphate-buffer solution and decapitated. Brains were removed from the skull and postfixed in the same fixative overnight. Subsequently, brains were cryoprotected in 20% sucrose overnight, frozen, and cut at 25 μm thickness on a cryostat (Leica CM3050 S; Leica Biosystems, Wetzlar, Germany). Sections were mounted on silane-coated glass slides (S9226; Matsunami Glass, Osaka, Japan). Sections were incubated with the primary antibodies overnight at room temperature. The following antibodies were used: anti-green fluorescent protein (GFP)

(1:200, goat polyclonal; Rockland Immunochemicals, Pottstown, PA, USA). For fluorescence microscopy, sections were treated with species-specific secondary antibodies conjugated to Alexa Fluor 488 (1:1000; Invitrogen, Carlsbad, CA, USA) and DAPI (1 mg/mL; Sigma-Aldrich, Saint Louis, MO, USA) for 2 h at room temperature. Fluorescence images were obtained using an all-in-one microscope (BZ-X710; Keyence, Osaka, Japan).

2.7 | Data analysis

All animals and samples were randomly assigned to the experimental groups. Investigators who collected and analyzed the data were not blinded to the conditions of the experiments. When the optic fiber position was not targeted correctly, we excluded those mice. Fiber photometry data were analyzed using custom-written programs in MATLAB. The YC ratio (a ratio of yellow to cyan fluorescence intensity; R) in one session was detrended using a cubic spline method and normalized within each trial by calculating the Z-score as $(R - R_{mean})/R_{SD}$, where R_{mean} and R_{SD} were the mean and standard deviation of the YC ratio for 5 s just before each trial start (TS).

2.8 | Statistics

We analyzed all data using custom codes written in MATLAB and showed the data as the mean ± standard error of the mean (SEM) unless otherwise described. Sample sizes, which were not predetermined but are similar to our previous reports,[8,45] are reported in the Results section and figure legends. To determine the statistical significance of differences, we performed paired t tests for two sample data sets (Figures 2C–F, 3A–D, 4C–E,G–I, Supplementary Figures S2 and S3). We confirmed the independence of two sample sets using a chi-squared test (Figure 4B,F). The null hypothesis was rejected when $p < 0.05$.

3 | RESULTS

3.1 | Subtle changes in DMS activity in the experts of action sequences

To monitor sequential behavior, we first trained mice with an FR1 task followed by an FR5 task (Figure 1A). We conducted fiber insertion surgery to mice prior to the FR1 task. In the FR1 task, mice were required to press a lever once to obtain a pellet reward. Mice acquired the action (lever press)-outcome (pellet) contingency in this task. After training at least 10 sessions of the FR1 task, the required number of lever presses was increased to five. The mice that experienced the first session in the FR5 task were categorized into a novice group, and the mice that had experienced seven sessions were categorized as experts. Mice occasionally suspended lever pressing and checked the empty food magazine before

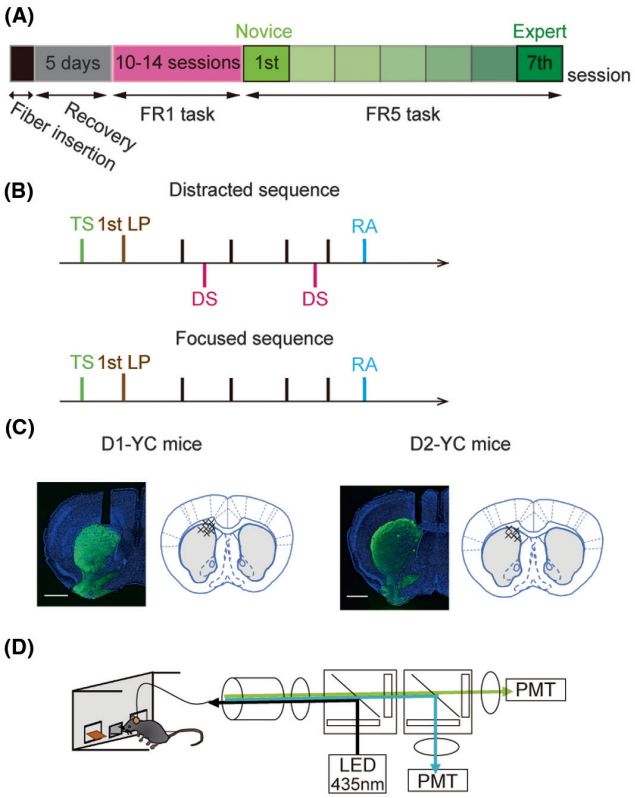

FIGURE 1 The experimental design. (A) The procedure of lever press training. First, mice were trained with the FR1 task and learned lever press–food reward contingency. Subsequently, the required number of lever presses was extended to five. The mice were categorized into a novice group that had experienced the first session and a group of experts that had trained for seven sessions. (B) Distracted sequences in which sequential lever pressing was suspended were occasionally induced between two consecutive lever presses (upper). In the FR5 task, mice had 4 opportunities for distraction. Contrast to distracted sequences, focused sequence has no checking behavior (bottom, 1st LP, first lever press; DS, distracted sequence; RA, reward acquisition; TS: trial start). (C) The histology data and schematic diagram showing the recording sites in D1- and D2-YC mice (scale bar = 1 mm). The implantation side was randomly assigned. (D) Schematic of the fiber photometry system. The fluorescence excitation and emission light path was through a single multimode fiber connected to the optical fiber cannula implanted in the dorsomedial striatum. A dichroic mirror was used to separate the fluorescence emission, and cyan and yellow fluorescence were corrected through bandpass filters and enhanced by photomultiplier tubes.

completing the 5 lever presses (Figure 1B upper). This checking behavior impeded the execution of the action sequence. We defined this obstruction for sequential behavior as a distracted sequence. The number of distracted sequences ranged from 0 to 4 within a trial. In contrast, we defined lever pressing without any checking as a focused sequence (Figure 1B bottom).

We recorded bulk Ca^{2+} activity in direct and indirect pathways in the DMS during mice performing the FR5 task to understand how the action sequence was regulated in the DMS (Figure 1C,D). The DMS has been segregated along with the anterior-posterior

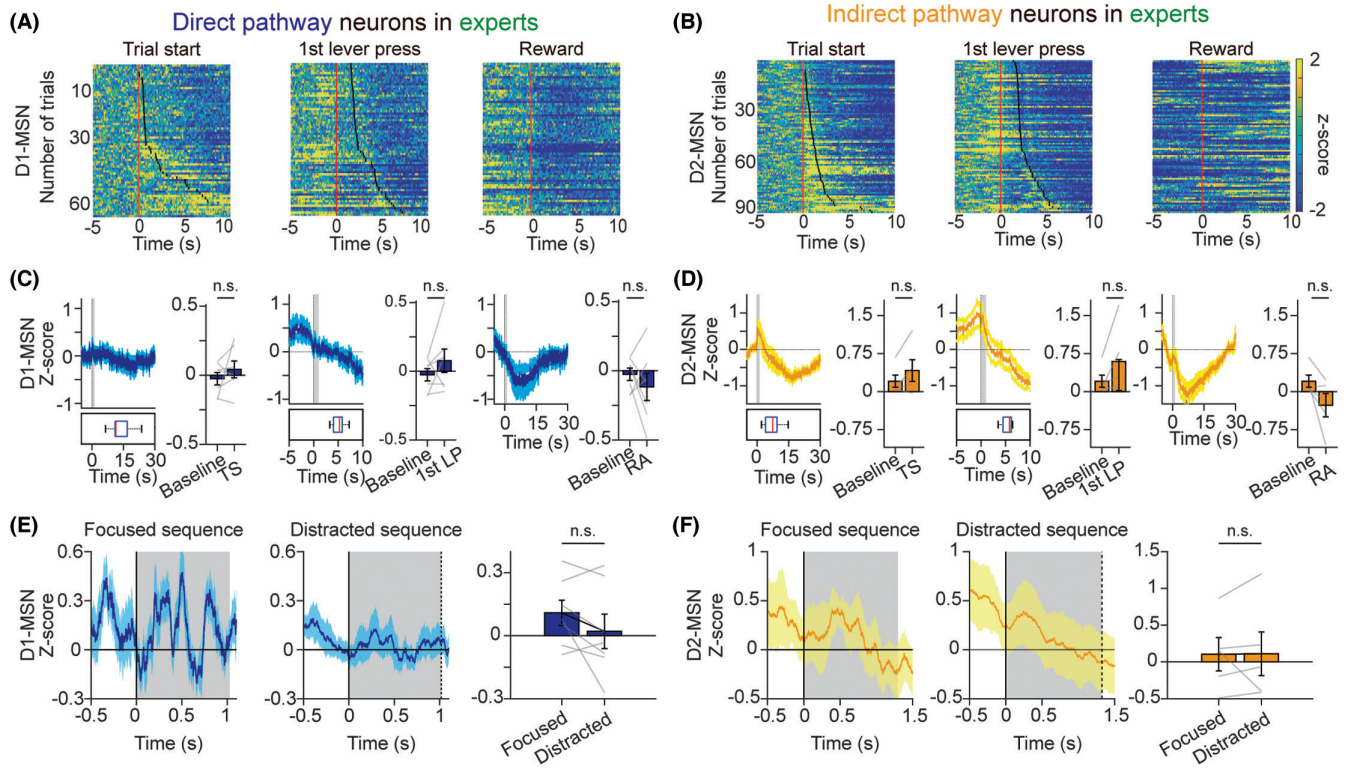

FIGURE 2 Dorsomedial striatum (DMS) activity in experts performing learned sequences. (A, B) Heatmaps of D1-MSN signals (A, 65 trials) and D2-MSN signals (B, 90 trials) in the DMS in one representative session aligned to the timing of trial start, first lever press, and reward acquisition, respectively. The heatmap is sorted by the latency from the trial start to the first lever press or the duration for five presses of the lever. (C, D) Averaged signals from all sessions of all D1-YC mice (C, $n=7$) and all D2-YC mice (D, $n=5$). The blue and orange lines indicate averaged signals, and the light blue and yellow areas represent ±SEM. Time 0 indicates the trial start, the first lever press, and reward acquisition. The shaded area represents from Time 0 to 1 s. Boxplots represent the latency from the trial start to the first lever press or the duration of 5 lever presses. The central red line indicates the median value. The bar charts compare the averaged activity for 1.0 s before the trial start (baseline) and after the trial start, first lever press, and reward acquisition. Comparing the activity from baseline and shaded area, respectively, there was no significant difference in neuronal activity for the 3 comparisons in D1-YC or D2-YC mice (C trial start: $t(6)=-0.96$, $p=0.37$, first lever press: $t(6)=-1.34$, $p=0.23$, reward acquisition: $t(6)=0.89$, $p=0.47$, D trial start: $t(4)=-1.90$, $p=0.13$, first lever press: $t(4)=-1.2$, $p=0.13$, reward acquisition: $t(4)=2.24$, $p=0.09$, paired t test). (E, F) Comparison of Ca^{2+} activity during focused and distracted sequences. The average activity (blue or orange) aligned with the timing of lever pressing was divided into 2 intervals; on the left is a focused sequence (mice pressed a lever sequentially), and on the right is a distracted sequence (mice checked the food magazine between two lever presses). D1-YC activity is E, and D2-YC activity is F. The dotted line shows the average time that mice entered the feeder box. The shaded area represents from Time 0 to 1 s. The bar charts compare the activity in shaded area during focused and distracted sequences. There was no noticeable difference in either group (D1-YC: $t(6)=1.62$, $p=0.16$, D2-YC: $t(4)=-0.05$, $p=0.96$, paired t test). n.s. $p \geqq 0.05$.

axes.[46,47] The fiber was implanted in D1 (direct pathway)-YC mice (Pde10a2-tTA::tetO-YCnano50; Adora2a-Cre triple-transgenic mice) or D2 (indirect pathway)-YC mice (Drd2-tTA::tetO-YCnano50 double-transgenic mice) to monitor the Ca^{2+} signals.[8]

The neuronal activity from the expert D1- and D2-YC mice exhibited a periodic pattern during the FR5 task that we characterized by aligning the photometry data to each of three trigger events (Figure 2A–D). Figure 2A,C represent D1-MSN activity, and Figure 2B,D show D2-MSN signals from the expert group. The expert D1-YC mice took 11.40±5.23 s from the trial start to the 1st lever press and 5.26±1.23 s from the 1st to 5th lever press, whereas the expert D2-YC mice required 7.47±4.28 s from the trial start to the 1st lever press and 5.82±1.06 s from the 1st to 5th lever press (Figure 2C,D box plots).

Comparing the signals from 1.0 s before the trial start and 1.0 s after three events, trial start, first lever press, and reward acquisition, there was no significant difference in all comparisons in either D1-MSN or D2-MSN activity (Figure 2C,D bar charts: C trial start: $t(6)=-0.96$, $p=0.37$, first lever press: $t(6)=-1.34$, $p=0.23$, reward acquisition: $t(6)=0.89$, $p=0.47$, D trial start: $t(4)=-1.90$, $p=0.13$, first lever press: $t(4)=-1.2$, $p=0.13$, reward acquisition: $t(4)=2.24$, $p=0.09$, paired t test). Next, we compared neuronal activity during focused and distracted sequence trials in expert mice. Because a focused trial did not include a distracted sequence, we applied the same time window used in the distracted sequence trial. We observed no significant differences between focused and distracted sequence trials in either D1-YC or D2-YC experts (Figure 2E,F: D1-YC: $t(6)=1.62$, $p=0.16$, D2-YC: $t(4)=-0.05$, $p=0.96$, paired t test).

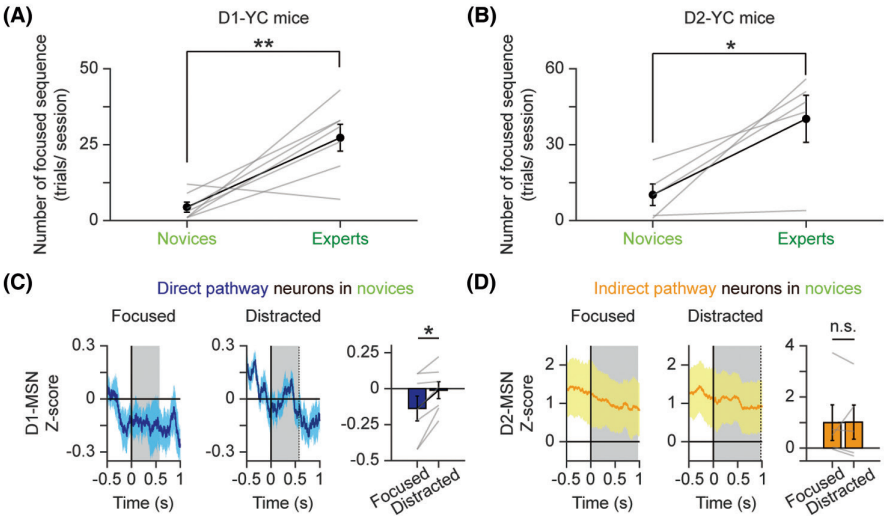

FIGURE 3 Dorsomedial striatum activity in the novices performing action sequences. (A, B) Comparison of the number of focused sequences between the novice and expert groups in both D1- (A) and D2-YC(B) mice (D1-YC mice: $t(6) = 5.04$, $p = 0.006$, D2-YC mice: $t(4) = 4.43$, $p = 0.03$, paired t test). It became clear that focused sequences increased significantly along with FR5 training in D1- and D2-YC mice. (C, D) Comparison of neuronal activity during the execution of focused and distracted sequences in the novice D1- (C) and D2-YC mice (D). The average activity (blue or orange) is aligned with the timing of lever pressing. The dotted line shows the average time that mice entered the feeder box. The shaded area exhibits from Time 0 to the average timing which mice entered the magazine. Comparing the activity in shaded areas, the activity in the novice D1-YC mice was increased more significantly during the distracted than the focused sequences, while the signals in the novice D2-YC mice were unchanged (D1-YC mice: $t(6) = -3.41$, $p = 0.01$, D2-YC mice: $t(4) = -0.08$, $p = 0.94$, paired t test). $^{*}p < 0.05$, $^{**}p < 0.01$, n.s. $p \geqq 0.05$.

3.2 | Sequence-related DMS activity in the novices of action sequences

Consistent with previous reports,[14,31] we observed only faint event-related activity in DMS neurons in mice who trained with sequential behavior repetitively. We then focused on analyzing the data when the animals were still beginners at performing sequential behaviors (Figure 1A). We found that compared to experts, novices displayed more sequence distraction (Figure 3A,B: D1-YC mice: $t(6) = 5.04$, $p < 0.001$, D2-YC mice: $t(4) = 4.43$, $p < 0.05$, paired t test). D1-MSN activity was inhibited during action sequences, and the inhibition was significantly weakened in trials where the sequence was distracted (Figure 3C: D1-YC mice: $t(6) = -3.41$, $p < 0.05$, paired t test). By contrast, the indirect pathway showed a slight increase in neural activity when the animals executed the action sequences (Figure 2D). However, the slight activation was not modulated by the trial type (Figure 3D: D2-YC mice: $t(4) = -0.08$, $p = 0.94$, paired t test).

3.3 | Optogenetic activation of the direct pathway promoted distracted sequence

To understand the function of sequence-related signaling in the DMS, we applied optogenetic manipulation to direct and indirect pathway neurons. We bilaterally enhanced the direct pathway activity using D1-ChR2 mice (Pde10a2-tTA::tetO-ChR2-YFP; Adora2a-Cre triple-transgenic mice) because direct pathway signals were inhibited during the focused sequences (Figure 3C), whereas indirect pathway activity was suppressed by using D2-ArchT mice (Drd2-tTA::tetO-ArchT-EGFP double-transgenic mice) because their activity slightly surpassed the baseline activity[8] (Figures 3D and S1). D1-ChR2 mice were illuminated with blue before yellow light to activate the direct pathway or with yellow light as a control at the time of the first lever press (Figure 4A left). D2-ArchT mice were illuminated with yellow light to inhibit the indirect pathway or with blue light such that they were insensitive to signals at the first lever press (Figure 4A right). We treated the same D1-ChR2 and D2-ArchT mice as controls using yellow and blue light, respectively.

In D1-ChR2 control mice, the percentage of distracted trials was 46% and of focused trials was 54%, whereas in the excitation group, the percentage of distracted trials was 68% and of focused trials was 32%. The chi-squared test revealed that stimulation of direct pathway activity affected sequential behavior (Figure 4B: $n = 6$ animals, $\chi^2(5) = 4.14$, $p < 0.05$, chi-squared test). The frequency of focused sequences within a session was significantly curbed by the excitation of direct pathway activity (Figure 4C left: $n = 6$ animals, percentage of focused trials: $t(5) = -3.72$, $p < 0.05$, paired t test). In addition, the frequency of distractions within a trial was heightened by this manipulation (Figure 4C right: $n = 6$ animals, frequency of distractions: $t(5) = 3.32$, $p < 0.05$, paired t test). These results indicated that enhanced direct pathway activity obstructed sequential lever pressing. Moreover, the time that mice spent in the food magazine was not modified, although the activation of the direct pathway urged mice to check the magazine frequently (Figure 4D: magazine dwell time: $t(5) = -0.12$, $p = 0.91$, paired t test). This activation made no difference in the proportion of completed trials in which mice

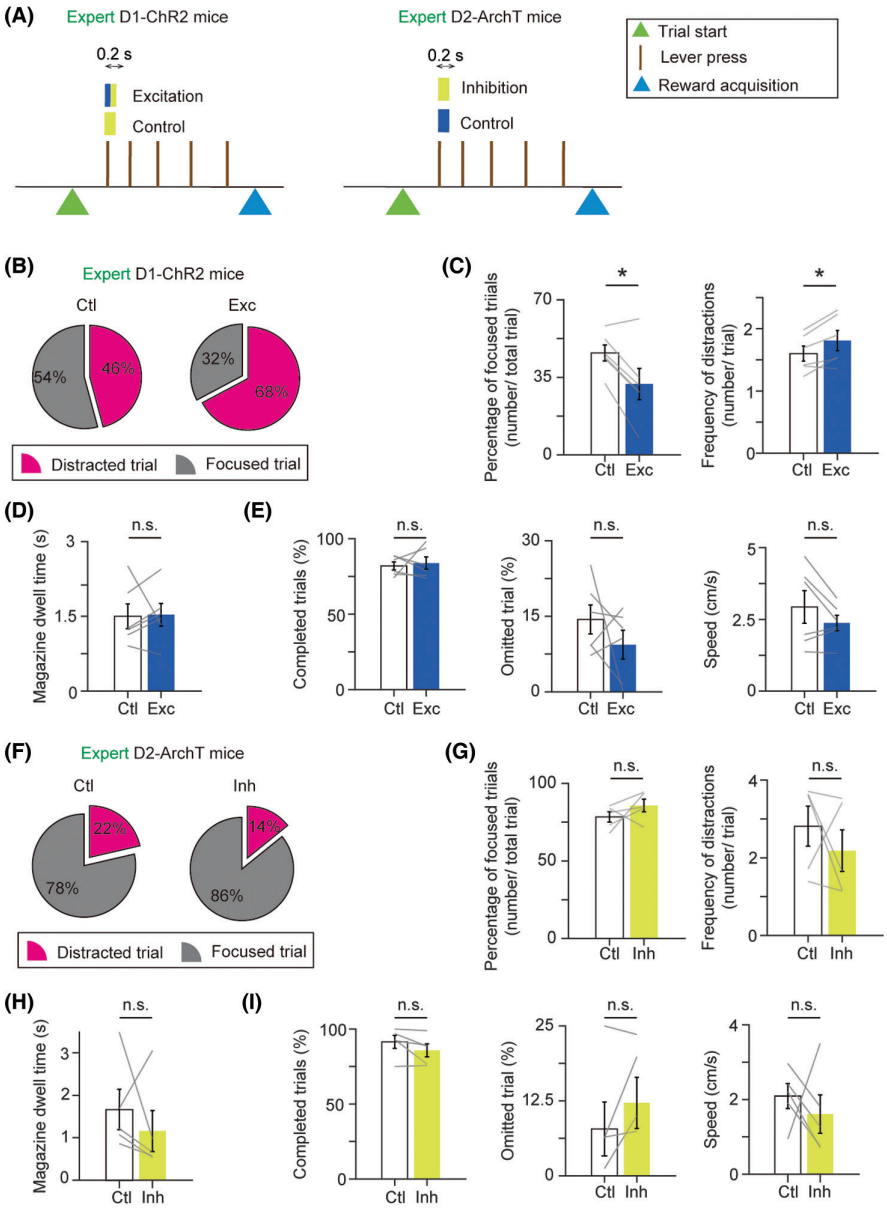

FIGURE 4 Optogenetic excitation of the direct pathway increased distracted sequences, while suppressing the indirect pathway did not impact sequential behavior. (A) Timing of illumination in the FR-5 schedule. At the first lever press, the D1-MSN was optogenetically activated (left), and the D2-MSN (right) was inhibited. To excite the structures, they were illuminated with blue light for 0.1 s before illumination with yellow light for 0.1 s. Illuminating with yellow light for 0.2 s rendered the structures insensitive. To suppress the structures, they were illuminated with yellow light for 0.2 s. Illuminating with blue light for 0.2 s rendered them insensitive. (B) The percentage of distracted (magenta) and focused trials (gray) per session in the control and excitation groups, respectively. In the focused trial, the mice pressed a lever 5 times consecutively. In the distracted trial, the mice checked the magazine at least once within a trial. The excitation of D1-MSN caused bias between these two groups. ($n = 6$ animals, $\chi^2(5) = 4.14$, $p = 0.04$, chi-squared test). (C) The percentage of focused trials within total trials (left) and the frequency of distraction number per trial in D1-ChR2 mice (right). The activated D1-MSN reduced focused trials (left) and increased distraction frequency per trial (right) ($n = 6$ animals, percentage of focused trials: $t(5) = -3.72$, $p = 0.01$, frequency of distractions: $t(5) = 3.32$, $p = 0.02$, paired t test). (D) The activated D1-MSN did not impact the magazine dwell time during which mice spent time around the food magazine ($n = 6$ animals, magazine dwell time: $t(5) = -0.12$, $p = 0.91$, paired t test). (E) The percentage of completed trials was unchanged, but the percentage of omitted trials was decreased by enhanced D1-MSN activity ($n = 6$ animals, completed trials: $t(5) = 0.35$, $p = 0.74$, omitted trials: $t(5) = 2.80$, $p < 0.05$, paired t test). The speed with which mice engaged in the FR5 task was not altered ($n = 6$ animals, speed: $t(5) = -1.70$, $p = 0.17$, paired t test). (F) Inhibiting the D2-MSN of D2-ArchT mice produced no bias between the control and inhibition groups ($n = 5$ animals, $\chi^2(4) = 1.84$, $p = 0.17$, chi-squared test). (G) Inhibiting the D2-MSN did not affect the percentage of focused trials per session or the frequency of distracted trials per trial ($n = 5$ animals, percentage of focused trials: $t(4) = 1.21$, $p = 0.29$, frequency of distracted trials: $t(4) = -0.86$, $p = 0.44$, paired t test). (H) Suppressing the D2-MSN did not affect the dwell time in the magazine ($n = 5$ animals, magazine dwell time: $t(4) = 0.46$, $p = 0.82$, paired t test). (I) Inhibiting the D2-MSN did not change the percentage of completed and omitted trials or the speed ($n = 5$ animals, completed trials: $t(4) = -1.82$, $p = 0.14$, omitted trials: $t(4) = 2.24$, $p = 0.089$, speed: $t(4) = 0.63$, $p = 0.56$, paired t test). *$p < 0.05$, n.s. $p \geqq 0.05$.

pressed a lever five times, regardless of whether they were focused or distracted when performing the sequence (Figure 4E left: $n=6$ animals, completed trial: $t(5)=0.35$, $p=0.74$, paired t test). In addition, the percentage of omitted trials, in which mice did not press a lever during lever presentation, was not modified by the activation of the direct pathway (Figure 4E middle: $n=6$ animals, omitted trials: $t(5)=-1.07$, $p=0.33$, paired t test). These outcomes suggested that the excitation of the direct pathway did not affect the memory of the task structure or motivation to perform the task. As it is widely accepted that the direct pathway promotes movement,[19,21] we investigated the speed that mice moved through the operant chamber during the task, and it was not altered between groups (Figure 4E right: $n=6$ animals, speed: $t(5)=-1.70$, $p=0.17$, paired t test). We also confirmed that the duration from mice pressing a lever to check the magazine, frequency of entry to the nonmagazine area, or the number of inactive lever presses was not changed by the manipulation (Figure S2A–C). All together, these results indicate that the strengthened direct pathway activity impeded sequential lever pressing while preserving motor function and memory of the task structure.

By contrast with the direct pathway activation, suppressing indirect pathway activity did not impact sequential lever pressing (Figure 4F: $n=5$ animals, $\chi^2(4)=1.84$, $p=0.17$, chi-squared test, 4G: percentage of focused trials: $t(4)=1.21$, $p=0.29$, frequency of distracted trials: $t(4)=-0.86$, $p=0.44$, paired t test, 4H: magazine dwell time: $t(4)=0.46$, $p=0.82$, paired t test). Moreover, inactivating the indirect pathway did not affect the proportion of completed and omitted trials or the speed with which mice engaged in the FR5 task (Figure 4I: $n=5$ animals, completed trials: $t(4)=-1.82$, $p=0.14$, omitted trials: $t(4)=1.51$, $p=0.21$, speed: $t(4)=0.63$, $p=0.56$, paired t test). Suppressing indirect pathway activity significantly prolonged the time from the lever-pressing zone to the food magazine, which we did not observe in the direct pathway activation experiment (Figure S2D). The frequency with which mice entered the nonmagazine area, the number of inactive lever presses or the completion latency from 1st to 5th lever pressing were not modified by suppressing the indirect pathway or activating the direct pathway (Figure S2E,F,G).

Based on these results, we proved that the direct pathway committed the execution of sequential behavior, while the involvement of the indirect pathway was not validated. Specifically, we clarified that inhibiting direct pathway neurons was essential for focused sequential lever pressing.

4 | DISCUSSION

Here, we examined whether and how DMS neurons regulate action sequences. Using cell-type-specific Ca^{2+} recording, we found that the direct pathway was inhibited when the mouse performed learned action sequences. The inhibitory signaling was vague in trials where the action sequences were disrupted. Optogenetic activation of the direct pathway at the time of sequence commencement promoted sequence distraction without changing motor functions. The indirect pathway was slightly activated at the sequence commencement, but the signaling was not correlated with focused or distracted sequences. Optogenetic inhibition of the indirect pathway did not change behavior. Together, these data suggest that the inhibition of the DMS direct pathway functions to execute action sequences, whereas the indirect pathway showed minor roles in the current lever-pressing task.

This study targeted the DMS anterior than bregma zero, because the posterior DMS has been argued to be involved in the auditory-dependent goal-oriented behavior.[48,49]

The DMS plays a pivotal role in forming action-outcome associations[16,32] and behavioral flexibility.[33,35,36] Its activity is known to be decreased along with repetitive training,[14,31,50] suggesting that DMS activity could interfere with the execution of already learned action sequences. Consistent with this idea, multiple studies,[12,37] including our present study, found inhibition of DMS activity in the execution of learned action sequences. Moreover, activation of the direct pathway induced unrewarded actions and caused the failure of sequences.[2] Thus, we predict that as the activity of the DMS seeks new action-outcome associations which enables the animals to acquire new learning when the action-outcome contingency has been changed (e.g., reversal learning),[51,52] inhibiting such activity could suppress the formation of new learning that prioritizes the execution of already learned sequences. However, it should be noted that the chronic ablation of the direct pathway could impair initiation of sequential behaviors.[2]

In the current study, we defined "distracted sequence" as a sequence interrupted by food magazine checking behavior. The optogenetic activation of the direct pathway increased the frequency of magazine checking behavior (Figure 3C), whereas other behaviors such as inactive lever press (Figure S2C) or entry to nonmagazine area (Figure S2B) were being intact. The result that the activation of the direct pathway specifically promoted the magazine checking behavior raised a possibility that the direct pathway signals reward anticipation and contributed to shortcutting the behavioral sequences toward reward acquisition. To test this hypothesis, we analyzed the photometry data of expert mice. If the direct pathway signaled reward anticipation, we should have observed the greater activity as the reward approaches, and the highest activity at the 5th lever press because the experts were well learned that the action triggers reward delivery in the FR5 task. However, the activity at the lever press was not changed across the number of lever presses (Figure S3). Moreover, there was no difference in the activities at 1st and 5th lever presses (Figure S3). These observations suggest that the DMS direct pathway does not signal reward anticipation at the timing of lever press.

Contrary to the marked functions of the direct pathway, we could not obtain obvious results from either observation or manipulation experiments in the indirect pathway. Optogenetic inhibition of the indirect pathway prolonged the duration between the lever-pressing zone to the food magazine entry (Figure S2D) which could suggest that suppression of the indirect pathway in the DMS

decease motivation. However, other indices involved in motivation such as % completed trials (Figure 4I left), % omitted trials (Figure 4I center), and latency to complete the sequence (Figure S2G) were not changed by the manipulation. Thus, the indirect pathway may give only a trivial effect on motivation in the current behavioral paradigm.

A previous study[12] found a unique population in the DMS, whose signals were activated at the start of the sequence, and such activation was correlated with disturbance of the sequence. In the current study, we observed a slight activation of the indirect pathway, but the signals were not correlated with behavior. The task used in the previous study required rats to press a lever five consecutive times, and no food was presented when the lever pressing was interrupted. It might be possible that the indirect pathway is engaged in action sequences which require the agent to be more focused. Another possibility is that the DMS indirect pathway is involved in the action sequences, as proposed by Geddes et al.[2] They developed a new sequence task that requires animals to switch their lever pressing left to right. Using this task, they observed that the indirect pathway in the dorsal striatum was activated during the switching and inactivation of the population-impaired sequence switch. Furthermore, Wang et al.[33] demonstrated that activation of the indirect pathway promoted switching to an already learned sequence, resulting in facilitation of reversal learning. Thus, it is also suggested that the DMS indirect pathway plays a role in switching the learned action sequences.

We have shown that inhibiting the DMS direct pathway is required to execute learned action sequences. Previous studies have suggested that various cortical regions are involved in encoding sub-elements of the sequence, such as sequence order and timing.[53-56] Further research is needed to determine how corticobasal ganglia circuits coordinate to control learned action sequences.

AUTHOR CONTRIBUTIONS

Conceptualization: Anna Kono, Kenji F. Tanaka, Katsunori Yamaura and Iku Tsutsui-Kimura. *Methodology*: Anna Kono. *Investigation*: Anna Kono, Kenji F. Tanaka and Iku Tsutsui-Kimura. *Visualization*: Anna Kono and Yu Shikano. *Funding acquisition*: Kenji F. Tanaka and Iku Tsutsui-Kimura. *Project administration*: Kenji F. Tanaka and Iku Tsutsui-Kimura. *Supervision*: Iku Tsutsui-Kimura. *Writing—original draft*: Anna Kono and Iku Tsutsui-Kimura. *Writing—review & editing*: Anna Kono, Yu Shikano, Kenji F. Tanaka, Katsunori Yamaura and Iku Tsutsui-Kimura.

ACKNOWLEDGMENTS

We thank Yuka Nakano, Norio Takata, and all Tanaka and Yamaura laboratory members. The completion of this research would not have been possible without the contributions and support of many individuals and organizations. We are deeply grateful to all those who played a role in the success of this project.

FUNDING INFORMATION

This work was supported by JST PRESTO (JPMJPR22S4), JST SPRING (JPMJSP2123), and AMED (JP19dm0207069).

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict interests.

DATA AVAILABILITY STATEMENT

The photometry data and the behavioral data are deposited at Dryad (https://doi.org/10.5061/dryad.0vt4b8h4f). Any MATLAB code used for analysis are available from the corresponding author upon request.

ETHICS STATEMENT

Approval of the Research Protocol by an Institutional Reviewer Board: N/A.

Informed Consent: N/A.

Registry and the Registration No. of the Study/Trial: N/A.

Animal Studies: All animal procedures were conducted following the National Institutes of Health Guide for the care and use of laboratory animals and approved by the Animal Research Committee of Keio University (Approval number: A2022-049).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Kono A, Shikano Y, Tanaka KF, Yamaura K, Tsutsui-Kimura I. Inhibition of the dorsomedial striatal direct pathway is essential for the execution of action sequences. Neuropsychopharmacol Rep. 2023;43:414–424. https://doi.org/10.1002/npr2.12369