

MIT Open Access Articles

Learning new sequential stepping patterns requires striatal plasticity during the earliest phase of acquisition

The MIT Faculty has made this article openly available. **Please share** how this access benefits you. Your story matters.

Citation: Nakamura, T., et al. "Learning New Sequential Stepping Patterns Requires Striatal Plasticity During the Earliest Phase of Acquisition." *Eur J Neurosci* 45 7 (2017): 901-11.

As Published: 10.1111/EJN.13537

Publisher: Wiley

Persistent URL: <https://hdl.handle.net/1721.1/133877>

Version: Author's final manuscript: final author's manuscript post peer review, without publisher's formatting or copy editing

Terms of use: Creative Commons Attribution-Noncommercial-Share Alike





Published in final edited form as:

Eur J Neurosci. 2017 April ; 45(7): 901–911. doi:10.1111/ejn.13537.

Learning New Sequential Stepping Patterns Requires Striatal Plasticity during the Earliest Phase of Acquisition

Toru Nakamura^{1,2}, Masatoshi Nagata¹, Takeshi Yagi¹, Ann M. Graybiel³, Tetsuo Yamamori^{2,4}, and Takashi Kitsukawa^{1,2,3}

¹KOKORO-Biology Group, Graduate School of Frontier Biosciences, Osaka University, Suita, Osaka, Japan

²Division of Brain Biology, National Institute for Basic Biology, Okazaki, Aichi, Japan

³Department of Brain and Cognitive Sciences and the McGovern Institute for Brain Research, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

⁴Laboratory of Molecular Analysis for Higher Brain Function, RIKEN Brain Science Institute, Wako, Saitama, Japan

Abstract

Animals including humans execute motor behavior to reach their goals. For this purpose, they must choose correct strategies according to environmental conditions and shape many parameters of their movements, including their serial order and timing. To investigate the neurobiology underlying such skills, we used a multi-sensor equipped, motor-driven running wheel with adjustable sequences of foothold pegs on which mice ran to obtain water reward. When the peg patterns changed from a familiar pattern to a new pattern, the mice had to learn and implement new locomotor strategies in order to receive reward. We found that the accuracy of stepping and the achievement of water reward improved with the new learning after changes in the peg-pattern, and c-Fos expression levels assayed after the first post-switch session were high in both dorsolateral striatum and motor cortex, relative to post-switch plateau levels. Combined *in situ* hybridization and immunohistochemistry of striatal sections demonstrated that both enkephalin-positive (indirect pathway) neurons and substance P-positive (direct pathway) neurons were recruited specifically after the pattern switches, as were interneurons expressing neuronal nitric oxide synthase. When we blocked N-methyl-D-aspartate (NMDA) receptors in the dorsolateral striatum by injecting the NMDA receptor antagonist, D-2-amino-5-phosphonopentanoic acid (AP5), we found delays in early post-switch improvement in performance. These findings suggest that the dorsolateral striatum is activated on detecting shifts in environment to adapt motor

Corresponding authors: Takashi Kitsukawa, Osaka University, 1-3 Yamada-oka, Suita, Osaka, 565-0871, Japan, Fax number: +81-6-6879-1922, kit@fbs.osaka-u.ac.jp; Ann M. Graybiel, Massachusetts Institute of Technology, 43 Vassar Street, 46-6133, Cambridge, MA 02139, +1-617-253-1599, graybiel@mit.edu.

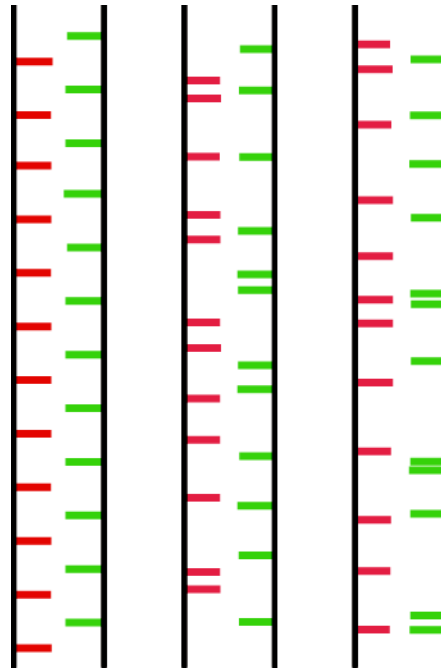
Competing Interest: The authors declare that there is no conflict of interest.

Author Contribution: T.N., Ta.Y., A.M.G., Te.Y., and T.K. designed research; T.N., M.N., and T.K. performed research; T.N. and T.K. analyzed data; and A.M.G. and T.K. wrote the paper.

Data Accessibility Statement: All data are available at: http://www.fbs.osaka-u.ac.jp/labs/yagi/NakamuraT/NakamuraData_2016.html.

behavior to the new context via NMDA-dependent plasticity, and that this plasticity may underlie forming and breaking skills and habits as well as to behavioral difficulties in clinical disorders.

Graphical abstract



Mice were trained to perform new, complex stepping patterns required by re-arranged peg-patterns in the step-wheel task. c-Fos expression demonstrated that striatal projection neurons of both direct and indirect pathways, together with nNOS-positive striatal interneurons, were activated in the first session with exposure to a new peg-pattern. Experiments with intrastriatal infusion of the NMDA blocker AP5 showed that the striatal plasticity was recruited at the earliest stage of learning.

Keywords

Mouse; Step-wheel; c-Fos; NMDA

Introduction

Most motor behaviors consist of sequences of movements. To improve these behaviors, successive movements must be executed in the correct order and with accurate timing. As such sequences are repeated, the movements become spatially and temporally more accurate by the process known as skill learning. With changing circumstances, however, we must discard or suppress old strategies and find new ones. Thus finding a new strategy is an important step for acquiring a new motor skill and for altering an old one.

The acquisition follows several phases: first, fast learning occurs in an early stage of learning, typically within a session; later, slower learning with smaller gains in performance

continues through further sessions. After a skilled behavior has been acquired, it can be retained even with some break in its repetition. The neural mechanisms underlying motor skill learning have been studied for decades in humans and experimental animals including non-human primates and rodents. Multiple cortical regions appear to be involved, including the motor cortex (Karni *et al.*, 1995; Muller *et al.*, 2002; Floyer-Lea & Matthews, 2005), and dorsolateral prefrontal (Sakai *et al.*, 1998) and premotor areas (Sakai *et al.*, 1998; Ghilardi *et al.*, 2000; Grafton *et al.*, 2002); and also both the basal ganglia (Doyon *et al.*, 2002; Floyer-Lea & Matthews, 2005; Lehericy *et al.*, 2005; Grol, 2006) and the cerebellum (Jenkins *et al.*, 1994; Sakai *et al.*, 1998; Eliassen *et al.*, 2001; Penhune & Doyon, 2002) exhibit increases or decreases in activity during motor learning, depending on the learning stages interrogated.

In non-human primates, neuronal activity in the pre-supplementary motor area decreases in the late stages of visuomotor sequence learning (Nakamura *et al.*, 1998; Sakai *et al.*, 1999). The activity of neurons in the anterior and posterior striatum has also been shown to depend on learning stage (Miyachi *et al.*, 2002), with the caudate nucleus and rostral putamen active in early stages of acquisition, and the caudal putamen active in the late stages. As well, spike activity at the beginning and end of movement sequences develops with experience (Desrochers *et al.*, 2015).

In rodents trained on rotarod tasks, changes in neuronal activity have been observed in the motor cortex and striatum (Costa *et al.*, 2004), and N-methyl-D-aspartate (NMDA) receptor function in the dorsolateral striatum has been found to be required (Lemay-Clermont *et al.*, 2011). Protein synthesis, required for many forms of synaptic plasticity, has been reported necessary in the motor cortex for inter-session improvement of motor performance of a skilled reach task (Luft, 2004).

Altogether, reaction times and success rate have been used for the evaluation of motor skill in most studies. Here we measured timing and accuracy of motor performance requiring patterned locomotion in a step-wheel system in which the mice could be trained to run in different stepping patterns by experimenter-controlled placement of pegs placed like rungs on a ladder to provide footholds (Kitsukawa *et al.*, 2011; Nakamura *et al.*, 2014). The stepping pattern of the mice could be changed by shifts in peg-patterns, requiring the mice to change running strategy. The timing of footfalls could be monitored at a millisecond level by touch sensors on each peg, allowing analysis of the accuracy and timing of the peg touches. We trained mice with an initial peg-pattern, and then, without warning, introduced a new pattern in order to force the animals to change their running patterns. We monitored motor cortical and, especially in detail, striatal activity, by analyzing the expression of the immediate early gene, c-Fos, in identified neurons in relation to striatal neuron type. Finally, we tested for the necessity of the striatal activity found by examining the effects of blockade of NMDA receptor activity in the dorsolateral striatum. Our findings demonstrate that flexible, rapid responses to changes imposed by the environment require NMDA-mediated plasticity in the dorsolateral striatum.

Materials and methods

Animals

Two lines of mice (10-20 weeks old) of different sizes, ICR (weighing 30 to 40 g, $n = 12$) and C57BL/6 (20 to 25 g, $n = 32$) mice, were used for this study. All mice were purchased from SLC Japan (Hamamatsu, Japan). ICR mice were used for motor learning experiments and immunostaining studies, and C57BL/6 mice were used for motor learning experiments with pharmacologic treatment. All procedures were performed in accordance with the guidelines for the conduct of animal experiments of Osaka University, National Institute for Basic Biology, and the Committee on Animal Care of the Massachusetts Institute of Technology. The mice were allowed free access to dry pellet food. During experimental periods, the mice were allowed to drink water in the wheel during and after training sessions. Additional water was given in their home cages as needed to reach 3 ml/day. They were given free access to water for an entire day every 1-2 weeks during training. Ad lib water was also given if their weight fell to less than 80% of its initial level.

The step-wheel task

The step-wheel (Fig. 1) consisted of an upright motor-driven rotating running wheel with ladder-like pegs for foot-holds for locomoting mice (Kitsukawa *et al.*, 2011). The diameter of the step-wheel was 32 cm, and the circumference was 100 cm. Varying the positions of the right and left pegs allowed control of the stepping patterns of the mice. A spout, from which the mouse could drink water as reward, protruded through a space between the right and left pegs. To monitor individual paw-touches to each peg, we applied ± 100 mV square-waves at 20 kHz. Paw-touches were detected by the reduction of the amplitude using a voltage sensor ('paw-touch sensor') line connected to each peg (Fig. 1A) and custom software developed with LabVIEW (National Instruments, TX). The applied voltage did not cause any aversive behavioral effect on mice (Kitsukawa *et al.*, 2011). Wheel rotation, peg passage and the position of the mouse were monitored with infrared photobeam units (Fig. 1B).

The running surface of the wheel was made up of spatially organized rung-like pegs. One trial of a given peg-pattern consisted of 24 pegs, 12 for the right foot and 12 for the left foot, and a given peg-pattern was repeated twice in one turn of the wheel. We exposed the mice to three different peg-patterns, termed A, C1 and C2 (Fig. 1C). One session typically consisted of 60 trials.

Timeline of training

1. Habituation: Initially, naïve mice deprived of water over night were placed in the stationary step-wheel with peg-pattern A for about 10 min, during which time they could find the spout and drink water. This habituation training typically took two days.
2. Pre-training: Wheel turning was begun during each session, still with peg-pattern A, with gradual increases in speed until the experimentally determined plateau of about 7.2 rpm for ICR mice and 6 rpm for C57BL/6 mice, corresponding to 12

cm/s and 10 cm/s on running surface, respectively. This pre-training period typically lasted about 3 weeks.

3. After the pre-training period, peg-patterns were changed without changing turning speed. Mice were trained once a day.

Accelerating wheel test used in pharmacologic experiments

For this test, to determine the appropriate concentration of D-2-amino-5-phosphonopentanoic acid (AP5, Sigma), an NMDA receptor antagonist (Fig. 6B), we ran mice on the peg-pattern A and gradually increased the turning speed of the wheel from 3.75 to 10 rpm (6.3 to 16.7 cm/s). The fastest turning speed at which the mice could stay near the spout as detected by a photobeam was measured 8-10 times, and the average of the three fastest turning speeds was used as the score for each mouse. The fastest turning speed that mice could run was determined by the absence of *photobeam a*-breaks, which indicates that mice were not close to the spout, for 3 s.

Two indices were used for the evaluation of motor skill learning: 1) the inter-touch variance in peg-touch times and 2) the water-position time. The inter-touch variance was calculated as the variances of time spent between two touches of pegs separated by 11 pegs (the inter-touch interval) and was used to evaluate the accuracy of stepping. In a session, the inter-touch intervals for every pair of pegs (e.g., peg 1 to peg 12) were calculated trial by trial. The inter-touch variance was the averaged variance of collected inter-touch intervals for every pair of pegs in a session. We calculated the inter-touch intervals only when mice ran near the spout, breaking photobeam *a*, because the inter-touch intervals should be quite different values when touches were recorded outside of the spout area. These values were calculated and then averaged over every right and left peg pair. The water-position time is the length of time that mice break the *photobeam a* in a given session. All data analysis was performed with custom software developed with MATLAB (Mathworks, MA).

Immunohistochemistry and *in situ* hybridization

Brains were removed from mice deeply anesthetized with sodium pentobarbital (200 mg/kg weight) 30 min after the onset of training and freshly frozen. Serial transverse sections (10- μ m thickness) were cut on a cryostat and collected on glass slides. Immunohistochemical detection of c-Fos protein was performed with anti-c-Fos antibody (1/1000 dilution; sc-52, Santa Cruz, CA). Signals were detected by horseradish peroxidase activity with 0.025% diaminobenzidine and 0.03% hydrogen peroxide.

In situ hybridization was conducted with digoxigenin-labeled antisense RNA probes. The RNA probes were transcribed from subcloned cDNA fragments. The cDNA fragments were obtained by RT-PCR from total mouse brain RNA. Primers used for the PCRs were agttcccttgggataacatc and tgagtcgtttcgcgaagtcc for preproenkephalin (enkephalin), tggacatggccagatctctc and cgcagtgacgttcgaacctg for preprotachykinin A (substance P), ggccatcactatattccctc and ccaccaggtgttaccagtgg for neuronal nitric oxide synthase, ttgcaggatgtcgatgacag and aggtcgtaaagggtctcttc for parvalbumin, aacatccaacagctcaccac and tctctgtaaactctctttg for calretinin, and tacaagcttctagctgtgag and tcactgagacggcggaatt for choline O-acetyltransferase.

Procedures for *in situ* hybridization followed the method developed by Schaeren-Wiemers and Gerfin-Moser (Schaeren-Wiemers & Gerfin-Moser, 1993). Sections were hybridized at 72°C for 12 hours, and signals were detected using alkaline phosphatase with nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate.

For combined immunohistochemistry and *in situ* hybridization, sections were first stained with anti-c-Fos antibody, and then treated for *in situ* hybridization. To protect mRNA in sections, yeast tRNA (0.2 mg/ml) was added to antibody solutions.

For quantitative analysis of labelled neurons, all analyses were carried out on blind-coded slides. Brain regions used for counting were determined according to the brain atlas by Paxinos and Franklin (2001) at the levels corresponding to atlas Figures 24 to 28 (antero-posterior = +0.86 to 0.38 mm from bregma). An ocular grid was used for the reference to determine sizes of the regions. At least three sections per animal were used.

Pharmacologic treatments

For implantation of cannulas, mice were deeply anesthetized with sodium pentobarbital (50 mg/kg) before surgery. C57BL/6 mice were used for the pharmacological experiments. Cannulas were inserted into the brain by stereotaxic technique (Paxinos & Franklin, 2001). The skin overlying the skull was incised, a small (< 1 mm in diameter) opening in the calvarium was made under stereotaxic guidance with a dental drill at coordinates corresponding to the right and left striatum (antero-posterior = +0.7 mm, medio-lateral = ± 2.1 mm relative to bregma), and guide cannulas were inserted into the dorsolateral striatum (depth = 2.7 mm relative to bregma). Mice were allowed to recover for 1 week before experimentation. Obdurators in the cannulas were left in place, and replaced by injection cannulas connected to Hamilton syringes for microinjection.

AP5 (Sigma, 0.05 μ g or 0.5 μ g in 0.2 μ l saline per site) was administered bilaterally into the dorsolateral striatum, 30 min before the session each day. Microinjection was made at a constant rate of 0.2 μ l/min using Hamilton syringes. Saline was injected in control mice.

For histological reconstructions of cannula placements, mice were deeply anesthetized with sodium pentobarbital (50 mg/kg weight) and perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer solution (PBS, pH 7.4). Brains were removed from the cranium, post-fixed in the fixative, and equilibrated with 30% sucrose in 0.1 M PBS. Frozen sections (40- μ m thickness) were cut with a sliding slicer.

Statistical analysis

All data were analyzed by one-factor ANOVA, one-factor repeated measures ANOVA, or two-factor mixed design ANOVA followed by two-tailed t test or *post hoc* Bonferroni test, and are presented as mean \pm SEM.

Results

Performance improvement in the step-wheel

We asked whether improvement of performance could be found in mice trained on a novel complex peg-pattern in the step-wheel. We first trained mice with an initial peg-pattern (pattern A) until mice could run as fast as 7.2 rpm (12 cm/s), and then changed the peg-patterns to a novel peg-pattern, pattern C1, without changing the rotational speed of the wheel. To evaluate motor skill learning, we measured 1) the inter-touch timing variance and 2) the water-position time. To analyze the improvement of motor skill in the domain of the accuracy of stepping, we calculated the inter-touch variance for sessions run after the peg-pattern change from A to C1. If a mouse could learn the pattern of peg sequences, then it is possible that timing to touch pegs would become more accurate as training proceeds. When the stepping is accurate over trials in a session, the time duration consumed between touches of two particular pegs would be similar in every trial and the variance of the duration over trials in a session, which was named as the inter-touch variance, would be small. The inter-touch variances with the novel peg-pattern, C1, were significantly smaller in sessions 8, 10, 11 and 12, relative to those in the first session (Fig. 2B, $n = 7$, one-factor repeated measures ANOVA, $F_{(11,66)} = 4.69$, $p = 2.9 \times 10^{-5}$; Bonferroni test, p values for sessions 2 to 12 were 1, 1, 1, 0.30, 0.11, 0.88, 0.017, 1, 0.037, 0.015, 0.008, respectively). This result indicated that over several sessions, the mice improved the accuracy of their stepping movements when confronted by a novel peg-pattern.

The water-position time was the length of time that mice could stay running in the vicinity of the spout, which was detected by the breakage of the *photobeam a*. The motivation of mice to run in the step-wheel was to be able to drink water from the water spout. Thus, the water-position time was considered as a measure of achievement. To keep drinking water, the mice were required to run at the same speed as the speed of the wheel, which was kept constant during experimental sessions. The water-position time thus reflected the ability of the mice to catch up the turning speed of the wheel as well. To maintain the high motivation level to allow the mice to run in the wheel, the turning speed was set so that mice could stay within the water-position region for at least about half of the total duration within a session. As a result, the water-position time was as high as 70% of session duration even in the first session (Fig. 2C). Nevertheless, the water-position times increased steadily, significantly rising in sessions 8, 10, 11 and 12 (Fig. 2C, $n = 7$, one-factor repeated measures ANOVA, $F_{(11,66)} = 3.86$, $p = 2.6 \times 10^{-4}$; Bonferroni test; p values for sessions 8, 10, 11 and 12 were 0.021, 0.010, 0.013 and 0.018, respectively). There was an interruption of 16 days between sessions 9 and 10, but this delay did not appear to alter the trends. In sessions 10-12, the inter-touch variances were as low (Fig. 2B), and the water-position times were as high (Fig. 2C) as those in session 8, suggesting that the motor ability, once learned in earlier sessions, was not lost by the interruption.

c-Fos expression in mice trained in the step-wheel

To identify brain regions potentially involved in the shaping of the sequential movement patterns observed after the peg-pattern change, we examined c-Fos expression in the brain, concentrating on the primary (M1) and secondary (M2) motor cortex and the dorsolateral

and dorsomedial striatum, by immunohistochemistry (Fig. 3). As the performance reached asymptotic levels at session 6, we compared the c-Fos expression of mice in sessions 1 and 7. We also counted the number of c-Fos-positive (c-Fos⁺) neurons in untrained caged (naïve) mice as a control. We found a remarkable increase in the number of c-Fos⁺ neurons in all regions examined in the trained mice ($n = 5$ for session 1 and $n = 5$ for session 7), relative to the naïve mice ($n = 5$), both in sessions 1 and 7, except for that in the dorsolateral striatum for session 7 (Fig. 4; M1: one-factor ANOVA, $F_{(2,12)} = 39.2$, $p = 5.5 \times 10^{-6}$; Bonferroni test, $p = 4.5 \times 10^{-5}$ vs session 1, $p = 7.3 \times 10^{-3}$ vs. session 7; M2: one-factor ANOVA, $F_{(2,12)} = 17.7$, $p = 3.0 \times 10^{-4}$; Bonferroni test, $p = 9.7 \times 10^{-4}$ vs. session 1, $p = 0.043$ vs. session 7; dorsolateral striatum: one-factor ANOVA, $F_{(2,12)} = 24.5$, $p = 5.8 \times 10^{-5}$; Bonferroni test, $p = 3.1 \times 10^{-4}$ vs. session 1, $p = 0.12$ vs. session 7; dorsomedial striatum: one-factor ANOVA, $F_{(2,12)} = 6.2$, $p = 0.01$; Bonferroni test, $p = 0.023$ vs. session 1, $p = 0.038$ vs. session 7). These differences in c-Fos expression between untrained naïve mice and trained mice most probably reflect neuronal activity related to motor activity. However, even among trained mice, differences in c-Fos expression were observed between sessions 1 and 7. Significantly more neurons expressed c-Fos in session 1 than in session 7 in the M1 (Fig. 4A, Bonferroni test, $p = 0.023$), M2 (Fig. 4B, Bonferroni test, $p = 0.029$), and dorsolateral striatum (Fig. 4C, Bonferroni test, $p = 2.7 \times 10^{-3}$), but not in the dorsomedial striatum (Fig. 4D, Bonferroni test, $p = 0.76$). These results may indicate that M1, M2 and dorsolateral striatum were involved in stage-dependent adaptation, such as motor learning, rather than solely motor activity.

To clarify neuronal types expressing c-Fos in the dorsolateral striatum, we used enkephalin (Enk) and substance P (SP) as respective markers for spiny projection neurons (SPNs) of the indirect (iSPNs, Enk-rich) and direct (dSPNs, SP-rich) pathways (Albin *et al.*, 1989; Kawaguchi *et al.*, 1995; Tepper *et al.*, 2010). We analyzed staining for c-Fos and each SPN-specific marker to identify iSPNs and dSPNs activated in the task (Fig. 5A-D).

c-Fos was strongly induced in both iSPNs (one-factor ANOVA, $F_{(2,12)} = 81.2$, $p = 1.1 \times 10^{-7}$; Bonferroni test, $p = 8.3 \times 10^{-4}$, $n = 5$) and dSPNs (one-factor ANOVA, $F_{(2,12)} = 47.7$, $p = 2.0 \times 10^{-6}$; Bonferroni test, $p = 6.5 \times 10^{-4}$) in the day of pattern-change, session 1, relative to session 7 (Fig. 5E). The ratio of c-Fos⁺ENK⁺ (iSPN) to c-Fos⁺SP⁺ (dSPN) neurons was not changed significantly between sessions 1 and 7 (Fig. 5E; two-tailed t test, $p = 0.70$), suggesting both direct and indirect pathways were activated in a balanced ratio regardless of learning stage.

We studied interneuron activity in the dorsolateral striatum by testing for parvalbumin (PV), neuronal nitric oxide synthase (nNOS), choline acetyltransferase and calretinin as markers for four different types of striatal interneurons. Strikingly, among these interneuron types, elevated c-Fos expression relative to levels in naïve control mice was detected only in PV⁺ and nNOS⁺ interneurons. Elevated c-Fos expression in PV⁺ interneurons was found both in sessions 1 and 7. There was no significant difference in the number of c-Fos⁺PV⁺ neurons between sessions 1 and 7, but these numbers were significantly higher than those for the naïve group (Fig. 5F; one-factor ANOVA, $F_{(2,12)} = 11.2$, $p = 0.002$; Bonferroni test, naïve vs. session 1, $p = 0.013$, naïve vs. session 7, $p = 0.039$, session 1 vs. session 7, $p = 0.42$). This result suggests that the activity of the PV⁺ interneurons could be involved in motor activity

per se rather than the learning of novel stepping. By contrast, for nNOS⁺ interneurons, the numbers of c-Fos⁺nNOS⁺ neurons was significantly higher in session 1 than the numbers in session 7 (Fig. 5G, one-factor ANOVA, $F_{(2,12)} = 20.7$, $p = 0.0001$; Bonferroni test, naïve vs. session 1, $p = 5.2 \times 10^{-4}$, naïve vs. session 7, $p = 0.16$, session 1 vs. session 7, $p = 0.037$).

Blockade of NMDA receptor in the striatum

The increase of c-Fos-expressing neurons in the first day of peg-pattern change could indicate that plastic changes were occurring at synapses in the striatum. In an attempt to test this possibility, we injected the NMDA receptor antagonist, AP5, directly into the dorsolateral striatum 30 min before the onset of the task (Fig. 6A) and examined performance in the step-wheel.

Before testing motor learning, we checked the effect on running performance of administering AP5 at each of two different doses (0.05 and 0.5 µg/site). We noted the highest speed that mice could run at each dose as the wheel turning rate increased (3.75 to 10 rpm) with the original peg-pattern A. Mice treated with the higher dose of AP5 (0.5 µg/site) could not catch up to wheel speeds above 6 rpm, whereas mice treated with the low dose of AP5 (0.05 µg/site) could run as well as mice treated with saline even when the speed was as high as 7.5 rpm (Fig. 6B). Accordingly, we administered the low dose of AP5 (0.05 µg/site) to both sides of the dorsolateral striatum at 30 min before the onset of each session and examined the inter-touch variances and the water-position times of the mice during new learning. The turning speed of the step-wheel was set to 6 rpm.

When the novel peg-pattern C2 was introduced, the inter-touch variances of both saline-treated and AP5-treated mice decreased gradually across successive sessions (Fig. 6C). Significant reduction was detected between session 1 and sessions 2-9 in saline-treated mice ($n = 6$, one-factor repeated measures ANOVA, $F_{(8,40)} = 15.54$, $p = 4.3 \times 10^{-10}$; Bonferroni test, p values for sessions 2-9 vs. session 1 were 0.016, 9.4×10^{-4} , 3.9×10^{-4} , 1.8×10^{-7} , 3.4×10^{-9} , 8.0×10^{-9} , 1.3×10^{-8} , 2.1×10^{-8} , respectively). In AP5-treated mice, the inter-touch variances decreased significantly from session 1 to sessions 3-9 ($n = 7$, one-factor repeated measures ANOVA, $F_{(8,48)} = 20.80$, $p = 3.6 \times 10^{-13}$; Bonferroni test, p values for sessions 2-9 vs. session 1 were 0.06, 1.0×10^{-5} , 8.5×10^{-8} , 1.7×10^{-9} , 4.7×10^{-11} , 2.1×10^{-11} , 2.7×10^{-11} , and 1.2×10^{-11} , respectively). The inter-touch variances of AP5-treated mice were higher than those of saline-treated mice in the first two sessions, but the changes did not reach statistical significance in any session.

We next analyzed the performance measured by the water-position time. Significant improvement was observed in sessions 3-9 both in the saline-treated mice ($n = 6$, one-factor repeated measures ANOVA, $F_{(8,40)} = 8.90$, $p = 6.6 \times 10^{-7}$; Bonferroni test, p values for sessions 2 to 9 were 0.057, 1.6×10^{-4} , 1.5×10^{-3} , 4.6×10^{-6} , 6.8×10^{-7} , 1.2×10^{-6} , 2.4×10^{-4} and 2.3×10^{-6} , respectively) and in the AP5-treated mice ($n = 7$, one-factor repeated measures ANOVA, $F_{(8,48)} = 32.74$, $p = 1.1 \times 10^{-16}$; Bonferroni test, p values for sessions 2 to 9 were 0.16, 4.3×10^{-8} , 7.2×10^{-11} , 4.1×10^{-12} , 1.6×10^{-13} , 8.5×10^{-14} , 2.8×10^{-13} and 2.1×10^{-13} , respectively), compared to values in the first session for each group (Fig. 6D). The water-position times of the AP5-treated mice were lower than those of the saline-treated mice in the first three sessions. Although a two-factor mixed design ANOVA did not

detect modulation by drug ($n = 13$, $F_{(8,99)} = 1.36$, $p = 0.25$), significant interactions were found between sessions and drug ($n = 13$, $F_{(8,99)} = 2.25$, $p = 0.03$). The difference between the two groups reached significance in the second session (two-tailed t test, p value for sessions 1-9 were 0.20, 0.016, 0.20, 0.38, 0.77, 0.60, 0.22, 0.43 and 0.38, respectively). These results indicated that mice treated with AP5 had difficulty in running on the novel pattern of pegs in early sessions.

We therefore tracked the changes of the performance of mice within individual sessions. For this purpose, we used the water-position time, because the water-position time had a discrete value for each trial, enabling us to divide a session into blocks of early and late trials, unlike the inter-touch variance for the calculation of which all the inter-touch intervals recorded within a session were used. Cumulative times spent in the water-position slowly increased in the first half of early sessions (Fig. 7A). This slow improvement was observed in both groups of mice in session 1, but was only observed in AP5-treated mice in session 2. In the third to last sessions, the cumulative water-position time increased linearly with trials within each session. To determine whether the rate of increases in the cumulative water-position times changes within a single session, we compared the slopes of growth rates between early (trials 1-15) and late (trials 31-45) trials (Fig. 7B). We restricted the late trials to trials 31 to 45, although mice typically ran 60 trials, because some mice drank water only intermittently after trial 50.

The slopes of the change in cumulative water-position times increased significantly in the saline-treated mice during early (Fig. 7C; $n = 6$, one-factor repeated measures ANOVA, $F_{(8,40)} = 12.76$, $p = 6.8 \times 10^{-9}$; Bonferroni test, p values for sessions 2-9 were 1.9×10^{-4} , 2.2×10^{-7} , 2.3×10^{-5} , 7.3×10^{-8} , 6.3×10^{-9} , 6.6×10^{-9} , 4.1×10^{-7} and 2.1×10^{-8} , respectively) and late (Fig. 7D; $F_{(8,40)} = 6.77$, $p = 1.4 \times 10^{-5}$; Bonferroni test, p values for sessions 2-9 were 1, 0.045, 3.8×10^{-3} , 2.1×10^{-4} , 1.3×10^{-4} , 3.6×10^{-5} , 9.0×10^{-3} and 1.4×10^{-4} , respectively) trials. Comparable increases were also observed in the AP5-treated mice during early ($n = 7$, one-factor repeated measures ANOVA, $F_{(8,48)} = 26.43$, $p = 4.2 \times 10^{-15}$; Bonferroni test, p values for sessions 2-9 were 0.10, 3.0×10^{-8} , 2.3×10^{-10} , 3.7×10^{-11} , 3.8×10^{-12} , 1.4×10^{-12} , 1.1×10^{-11} and 4.0×10^{-12} , respectively) and late (one-factor repeated measures ANOVA, $F_{(8,48)} = 29.45$, $p = 5.6 \times 10^{-16}$; Bonferroni test, p values for sessions 2-9 were 0.23, 5.7×10^{-7} , 3.7×10^{-9} , 6.6×10^{-11} , 6.6×10^{-13} , 2.7×10^{-13} , 4.0×10^{-13} and 7.6×10^{-14} , respectively) trials.

A two-factor mixed design ANOVA showed significant interaction between sessions and drug in early trials (Fig. 7C, $n = 13$, $F_{(8,99)} = 2.51$, $p = 0.016$) but not in later trials (Fig. 7D, $F_{(8,99)} = 0.81$, $p = 0.59$). The slopes of early trial cumulative curves for the AP5-treated mice were low in the early sessions (1-3). Compared to values for the saline-treated controls, slopes for the AP5-treated mice were lower, but the difference was significant only for the second session (Fig. 7C, two-tailed t test, p value for sessions 1-9 were 0.25, 9.6×10^{-3} , 0.11, 0.37, 0.93, 0.94, 0.36, 0.61 and 0.61, respectively). Statistically significant difference was not detected in the first session, probably because the slopes of both groups were low. The saline-treated mice reached an apparent plateau level in the second session, whereas the AP5-treated mice reached plateau in the third session. This difference may account for the fact that the significant difference was only observed in the second session. Collectively,

these findings show that the AP5-treated mice had more difficulty than the saline-treated controls in improving their early-session performance.

Discussion

Our findings demonstrate that new sequence learning can be monitored in detail in the step-wheel locomotor task and that acquisition rates exhibit early and late stages that can be identified by the different inter-footfall temporal variances and the different times spent close to the reward. Neuronal activity estimated by c-Fos expression was high in the motor cortex and dorsolateral striatum in the first session of new learning, relative to those in later sessions, and both direct and indirect pathway striatal projection neurons as well as nNOS-positive striatal interneurons exhibited characteristic changes from early to late in learning. New learning in the earliest phase was selectively slowed by blockade of NMDA receptors in the dorsolateral striatum. We conclude that conjoint activity of direct and indirect pathway neurons and local network neurons in the dorsolateral striatum occurs with new locomotor sequence learning and that this plasticity is likely critical for at least the earliest phases of new learning.

Motor skill learning in the step-wheel

Both inter-touch variances, used as a measure for accuracy, and water-position time, used as a measure for speed and achievement, improved as the training proceeded, and the acquired levels were maintained without practice for at least 16 days. These results are in accordance with features commonly found for motor skill: they are learned slowly and retained for extended times even without practice. We observed improvement of these indices with both ICR and C57BL/6 mice, indicating that the step-wheel could be used for assessment of motor behavior of mice of different sizes.

Our findings clearly indicate that the step-wheel has the potential for measurement of spatiotemporal motor accuracy, a direct measure of motor skills, in mice. Typical behavioral tasks used for rodents rarely test both motor accuracy and achievement. For example, the rotarod task, which is commonly used to estimate motor ability of rodents, uses time to fall from a rod, which does not directly measure motor accuracy but, rather, measures achievement.

Striatal regions activated in the step-wheel task

We found more c-Fos-positive neurons in the M1, M2 and dorsolateral striatum in the first session than in the seventh session. In the striatum, we observed c-Fos expression in striatal projection neurons (dSPNs and iSPNs), and two classes of striatal interneuron (PV⁺ and nNOS⁺ neurons). c-Fos expression was induced in both Enk⁺ iSPNs and SP⁺ dSPNs, and for both, the numbers of neurons expressing c-Fos were higher in the first session than in the seventh session. The ratio of c-Fos⁺Enk⁺ and c-Fos⁺SP⁺ neurons, however, did not change between the first and seventh sessions, which could indicate that the direct and indirect pathways are activated in a certain balance during running in the wheel regardless of learning stage.

The activation of the direct and indirect pathways is classically considered to elicit opposite effects (Albin *et al.*, 1989; Alexander and Crutcher, 1990), but they are also cooperative (Mink, 1996; Tecuapetla *et al.*, 2014; Oldenburg and Sabatini, 2015). Selective blockade (Hikida *et al.*, 2010) and selective activation (Kravitz *et al.*, 2012) of these two pathways in mice suggest that they can contribute to opposite effects in shaping behavior, the direct pathway working to associate rewarding stimuli and the indirect pathway working to avoid aversive stimuli. A human study using Parkinson's disease patients with or without medication executing cognitive reinforcement tasks supports this model (Frank *et al.*, 2004).

Both neuron types are concomitantly activated on the initiation of sequential movements, but are modulated differentially during the sequences (Cui *et al.*, 2014; Jin *et al.*, 2014). c-Fos expression is known to be differentially regulated in dSPNs and iSPNs both by cortical inputs and by dopamine (Gerfen & Suemeier, 2011). Thus, the balance we observed between these pathways was actively maintained at a population level likely by the cortical and dopaminergic systems.

cFos was also induced in two classes of striatal interneuron among those examined, PV⁺ and nNOS⁺. c-Fos⁺PV⁺ neurons were numerous, but their numbers were similar for the first and seventh sessions. We were thus unable to detect with this c-Fos method their possible modulation. We found, however, that the number of c-Fos expressing nNOS⁺ neurons was higher at the end of the first session than at the end of the seventh session. The activity of nNOS, the enzyme that produces nitric oxide (NO), is controlled by NMDA receptors (Kendrick *et al.*, 1996; Sammut *et al.*, 2007; Park & West, 2009) and dopamine D1 receptors (Park & West, 2009), and striatal NO is reported to enhance the up-state of striatal projection neurons *in vivo* (West & Grace, 2004). These facts may suggest that c-Fos expression in nNOS⁺ neurons indicates the possibility of interaction of glutamatergic inputs from the motor cortices and dopaminergic inputs in the first session, which together might contribute to maintain an active balance between the direct and indirect pathways. In addition, NO has been reported to induce striatal long-term depression at the cortical synapses on these neurons (Calabresi *et al.*, 1999; Rafalovich *et al.*, 2015). Our behavioral findings suggest that nNOS⁺ neurons could have participated in the plasticity that we observed behaviorally.

Plasticity in the striatum in the early stage of peg-pattern change

Blockade of NMDA receptors impaired the performance of mice in early trials in early sessions, as detected by their water-position times, suggesting that NMDA-dependent plasticity in the dorsolateral striatum was necessary for the earliest stages of the new learning. This result contrasts with the prior report that blockade of NMDA receptors in the dorsolateral striatum of mice running in the accelerating rotarod task led to deterioration of learning in the late acquisition stages, although some effects were observed by day 3 (Lemay-Clermont *et al.*, 2011). In addition, NMDA current was found to be increased in the late stages of the rotarod task (Yin *et al.*, 2009). Critically, task demands are quite different in the rotarod and step-wheel tasks. The step-wheel task requires the mice sequentially to touch the pegs in certain positions and at certain times as they run. This fine-tuning might have elongated the learning process, so that a comparably 'late' stage was not reached, but the step-wheel learning curves reached plateaus in late sessions.

Second, the primary motivation of mice performing the two tasks was likely quite different. The rotarod performance is aversion-driven, whereas step-wheel performance is reward-driven. Given that direct pathway neurons are in responding to reward and indirect pathway neurons referentially for aversive cues (Frank *et al.*, 2004; Hikida *et al.*, 2010; Kravitz *et al.*, 2012), and that these neurons are differentially modulated by dopamine, different striatal circuit participation could occur in the two tasks. Our findings emphasize that plastic changes become engaged in very early stage of motor skill learning (Wickens *et al.*, 1996; Surmeier *et al.*, 2009).

Third, changes in behavioral strategy and patterning were required for the step-wheel task but likely less so for the accelerating rotarod task, in which mice always employ a single global strategy, catching up to increasing speed of rotation, even though they could shape their footing through training (Cao *et al.*, 2015). In the step-wheel task, after the peg-pattern change, mice needed to change their running patterns in order to receive reward. It was in this early period, just after the peg-pattern change, that the mice should have changed their strategy and running patterns. The delay in post-shift performance in the AP5-treated mice may be caused by a failure in changing strategies.

It is well known that neuronal activity in the striatum changes over the course of motor learning. In the dorsolateral (sensorimotor) striatum, the majority of neurons were found to be preferentially active in the later stages of a visuomotor task in non-human primates (Miyachi *et al.*, 2002) and the accelerating rotarod task (Yin *et al.*, 2009), indicating that the dorsolateral striatum may contribute to the late stage of the motor skill learning. During T-maze over-training, the ensemble activity of dorsolateral striatal neurons gradually take on more stereotyped patterns emphasizing task beginning and ending (Jog *et al.*, 1999; Barnes *et al.*, 2005; Thorn *et al.*, 2010; Smith & Graybiel, 2013). However, ensembles of neurons that had been quieted when reward was withheld after the long training period could become active in the very early stage of reward reinstatement (Barnes *et al.*, 2005; Kubota *et al.*, 2009; Smith and Graybiel, 2016). This shift of active neurons suggests that the striatum, in the early stage, might cancel a set of active neurons established in the previous condition and explore a suitable set of active neurons for a new condition from a variety of neuron sets, and then rapidly form a new set of active neurons. In this exploitation and following exploratory formation of neuronal assemblies, synaptic weights likely change in the striatum, including those requiring the activity of NMDA receptors.

The striatum is thought to code action value (e.g., Samejima *et al.*, 2005). When executed movements are not suitable to achieve behavioral objective, animals cannot receive otherwise expected rewards, resulting in reward prediction errors. The dorsolateral striatum is thought to receive reward prediction error signals from dopamine-containing neurons in the substantia nigra, and likely other sources. In AP5-treated mice, water-position times were reduced in the first halves of trials during early sessions, when the reward prediction errors should have been large. The water-position times were reduced also in the control mice in the first session, but they exhibited quick recovery unlike the AP5-treated mice. NMDA-dependent plasticity may have occurred quickly according to the prediction error/loss of water reward in these controls.

It is reasonable to change behavior when its action value is lost, but behavioral change is slow or even nearly absent in some clinical conditions that include repetitive behavioral features, in addictive states and also in normal habits, in all of which neuroplasticity in the striatum may be insufficient. Future work on plasticity occurring in the very early stages of motor skill learning may open new therapeutic approaches for motor and psychomotor conditions and new protocols to break problem habits.

Acknowledgments

We thank the members of our laboratories for assistance and discussion. We are grateful to Henry Hall, Dr. Yasuo Kubota and Dr. Dan Hu of the Graybiel laboratory for their help in developing the step-wheel system. This work was supported by Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (25430009, 16H01613, T.K.), National Institute of Mental Health (R01 MH060379, A.M.G.) and Scientific Research on Innovative Areas (Neocortical Organization) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (22123009, Te.Y.).

References

- Albin RL, Young AB, Penney JB. The functional anatomy of basal ganglia disorders. *Trends Neurosci.* 1989; 12:366–375. [PubMed: 2479133]
- Alexander GE, Crutcher MD. Functional architecture of basal ganglia circuits - neural substrates of parallel processing. *Trends Neurosci.* 1990; 13:266–271. [PubMed: 1695401]
- Barnes TD, Kubota Y, Hu D, Jin DZ, Graybiel AM. Activity of striatal neurons reflects dynamic encoding and recoding of procedural memories. *Nature.* 2005; 437:1158–1161. [PubMed: 16237445]
- Calabresi P, Gubellini P, Centonze D, Sancesario G, Morello M, Giorgi M, Pisani A, Bernardi G. A critical role of the nitric oxide/cGMP pathway in corticostriatal long-term depression. *J Neurosci.* 1999; 19:2489–2499. [PubMed: 10087063]
- Cao VY, Ye Y, Mastwal S, Ren M, Coon M, Liu Q, Costa RM, Wang KH. Motor learning consolidates Arc-expressing neuronal ensembles in secondary motor cortex. *Neuron.* 2015; 86:1385–1392. [PubMed: 26051420]
- Costa R, Cohen D, Nicoletis M. Differential corticostriatal plasticity during fast and slow motor skill learning in mice. *Curr Biol.* 2004; 14:1124–1134. [PubMed: 15242609]
- Cui G, Jun SB, Jin X, Pham MD, Vogel SS, Lovinger DM, Costa RM. Concurrent activation of striatal direct and indirect pathways during action initiation. *Nature.* 2014; 494:238–242.
- Desrochers TM, Graybiel AM, Desrochers TM, Amemori K, Graybiel AM. Habit learning by naive macaques is marked by response sharpening of striatal neurons representing the cost and outcome of acquired action sequences. *Neuron.* 2015; 87:853–868. [PubMed: 26291166]
- Doyon J, Song AW, Karni A, Lalonde F, Adams MM, Ungerleider LG. Experience-dependent changes in cerebellar contributions to motor sequence learning. *Proc Natl Acad Sci U S A.* 2002; 99:1017–1022. [PubMed: 11805340]
- Eliassen JC, Souza T, Sanes JN. Human brain activation accompanying explicitly directed movement sequence learning. *Exp Brain Res.* 2001; 141:269–280. [PubMed: 11715072]
- Floyer-Lea A, Matthews PM. Distinguishable brain activation networks for short- and long-term motor skill learning. *J Neurophysiol.* 2005; 94:512–518. [PubMed: 15716371]
- Frank MJ, Seeberger LC, O'Reilly RC. By carrot or by stick: cognitive reinforcement learning in parkinsonism. *Science.* 2004; 306:1940–1943. [PubMed: 15528409]
- Gerfen CR, Surmeier DJ. Modulation of striatal projection systems by dopamine. *Annu Rev Neurosci.* 2011; 34:441–466. [PubMed: 21469956]
- Ghilardi MF, Ghez C, Dhawan V, Moeller J, Mentis M, Nakamura T, Antonini A, Eidelberg D. Patterns of regional brain activation associated with different forms of motor learning. *Brain Res.* 2000; 871:127–145. [PubMed: 10882792]

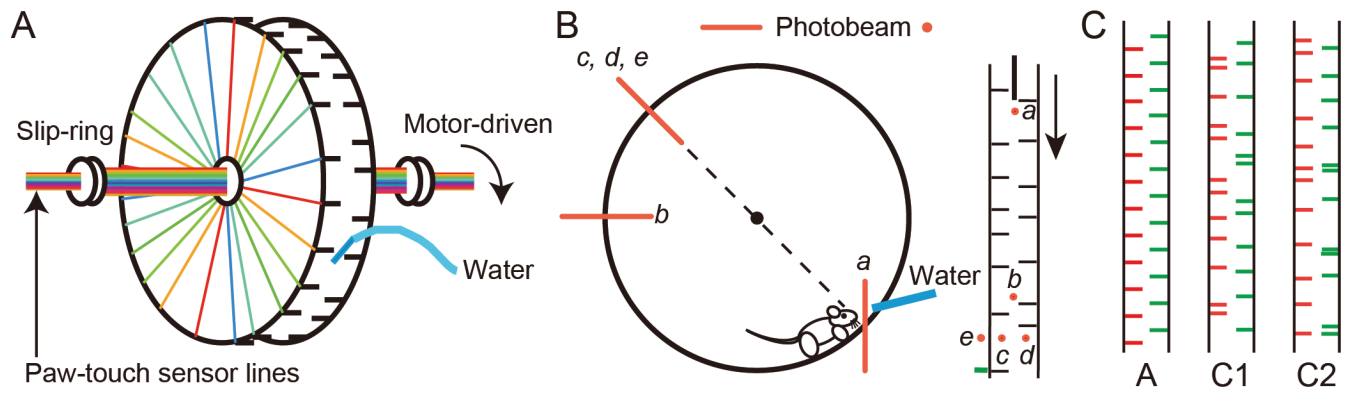
- Grafton ST, Hazeltine E, Ivry RB. Motor sequence learning with the nondominant left hand: A PET functional imaging study. *Exp Brain Res*. 2002; 146:369–378. [PubMed: 12232693]
- Grol MJ. Cerebral changes during performance of overlearned arbitrary visuomotor associations. *J Neurosci*. 2006; 26:117–125. [PubMed: 16399678]
- Hikida T, Kimura K, Wada N, Funabiki K, Nakanishi S. Distinct roles of synaptic transmission in direct and indirect striatal pathways to reward and aversive behavior. *Neuron*. 2010; 66:896–907. [PubMed: 20620875]
- Jenkins IH, Brooks DJ, Nixon PD, Frackowiak RS, Passingham RE. Motor sequence learning: a study with positron emission tomography. *J Neurosci*. 1994; 14:3775–3790. [PubMed: 8207487]
- Jin X, Tecuapetla F, Costa RM. Basal ganglia subcircuits distinctively encode the parsing and concatenation of action sequences. *Nat Neurosci*. 2014; 17:423–430. [PubMed: 24464039]
- Jog MS, Kubota Y, Connolly CI, Hillegaart V, Graybiel AM. Building neural representations of habits. *Science*. 1999; 286:1745–1749. [PubMed: 10576743]
- Karni A, Meyer G, Jezzard P, Adams MM, Tuner R, Ungerleider LG. Functional MRI evidence for adult motor cortex plasticity during motor skill learning. *Lett to Nat*. 1995; 377:155–158.
- Kawaguchi Y, Wilson CJ, Augood SJ, Emson PC. Striatal interneurons: chemical, physiological and morphological characterization. *Trends Neurosci*. 1995; 18:527–535. [PubMed: 8638293]
- Kendrick KM, Guevara-Guzman R, De La Riva C, Christensen J, Østergaard K, Emson PC. NMDA and kainate-evoked release of nitric oxide and classical transmitters in the rat striatum: In vivo evidence that nitric oxide may play a neuroprotective role. *Eur J Neurosci*. 1996; 8:2619–2634. [PubMed: 8996812]
- Kitsukawa T, Nagata M, Yanagihara D, Tomioka R, Utsumi H, Kubota Y, Yagi T, Graybiel AM, Yamamori T. A novel instrumented multipeg running wheel system, Step-Wheel, for monitoring and controlling complex sequential stepping in mice. *J Neurophysiol*. 2011; 106:479–487. [PubMed: 21525375]
- Kravitz AV, Tye LD, Kreitzer AC. Distinct roles for direct and indirect pathway striatal neurons in reinforcement. *Nat Neurosci*. 2012; 15:816–818. [PubMed: 22544310]
- Kubota Y, Liu J, Hu D, DeCoteau WE, Eden UT, Smith AC, Graybiel AM. Stable encoding of task structure coexists with flexible coding of task events in sensorimotor striatum. *J Neurophysiol*. 2009; 102:2142–2160. [PubMed: 19625536]
- Lehéricy S, Benali H, Van de Moortele PF, Pélégriani-Issac M, Waechter T, Ugurbil K, Doyon J. Distinct basal ganglia territories are engaged in early and advanced motor sequence learning. *Proc Natl Acad Sci U S A*. 2005; 102:12566–12571. [PubMed: 16107540]
- Lemay-Clermont J, Robitaille C, Auberson YP, Bureau G, Cyr M. Blockade of NMDA receptors 2A subunit in the dorsal striatum impairs the learning of a complex motor skill. *Behav Neurosci*. 2011; 125:714–723. [PubMed: 21859173]
- Luft AR. Motor skill learning depends on protein synthesis in motor cortex after training. *J Neurosci*. 2004; 24:6515–6520. [PubMed: 15269262]
- Mink JW. The basal ganglia: Focused selection and inhibition of competing motor programs. *Prog Neurobiol*. 1996; 50:381–425. [PubMed: 9004351]
- Miyachi S, Hikosaka O, Lu X. Differential activation of monkey striatal neurons in the early and late stages of procedural learning. *Exp Brain Res*. 2002; 146:122–126. [PubMed: 12192586]
- Muller RA, Kleinhans N, Pierce K, Kemmotsu N, Courchesne E. Functional MRI of motor sequence acquisition: Effects of learning stage and performance. *Cogn Brain Res*. 2002; 14:277–293.
- Nakamura K, Sakai K, Hikosaka O. Neuronal activity in medial frontal cortex during learning of sequential procedures. *J Neurophysiol*. 1998; 80:2671–2687. [PubMed: 9819272]
- Nakamura T, Sato A, Kitsukawa T, Momiyama T, Yamamori T, Sasaoka T. Distinct motor impairments of dopamine D1 and D2 receptor knockout mice revealed by three types of motor behavior. *Front Integr Neurosci*. 2014; 8:56. [PubMed: 25076876]
- Oldenburg IA, Sabatini BL. Antagonistic but not symmetric regulation of primary motor cortex by basal ganglia direct and indirect pathways. *Neuron*. 2015; 86:1174–1181. [PubMed: 26050037]
- Park DJ, West AR. Regulation of striatal nitric oxide synthesis by local dopamine and glutamate interactions. *J Neurochem*. 2009; 111:1457–1465. [PubMed: 19799710]

- Paxinos, G., Franklin, KBJ. *Mouse Brain in Stereotaxic Coordinates*. 2nd. Academic Press; San Diego: 2001.
- Penhune VB, Doyon J. Dynamic cortical and subcortical networks in learning and delayed recall of timed motor sequences. *J Neurosci*. 2002; 22:1397–1406. [PubMed: 11850466]
- Rafalovich IV, Melendez AE, Plotkin JL, Tanimura A, Zhai S, Surmeier DJ. Interneuronal nitric oxide signaling mediates post-synaptic long-term depression of striatal glutamatergic synapses. *Cell Reports*. 2015; 13:1336–1342. [PubMed: 26549446]
- Sakai K, Hikosaka O, Miyauchi S, Sasaki Y, Fujimaki N, Pütz B. Presupplementary motor area activation during sequence learning reflects visuo-motor association. *J Neurosci*. 1999; 19:RC1. [PubMed: 10234047]
- Sakai K, Hikosaka O, Miyauchi S, Takino R, Sasaki Y, Pütz B. Transition of brain activation from frontal to parietal areas in visuomotor sequence learning. *J Neurosci*. 1998; 18:1827–1840. [PubMed: 9465007]
- Samejima K, Doya K, Ueda Y, Kimura M. Representation of action-specific reward values in the striatum. *Science*. 2005; 310:1337–1340. [PubMed: 16311337]
- Sammur S, Park DJ, West AR. Frontal cortical afferents facilitate striatal nitric oxide transmission in vivo via a NMDA receptor and neuronal NOS-dependent mechanism. *J Neurochem*. 2007; 103:1145–1156. [PubMed: 17666041]
- Schaeren-Wiemers N, Gerfin-Moser A. A single protocol to detect transcripts of various types and expression levels in neural tissue and cultured cells: in situ hybridization using digoxigenin-labelled cRNA probes. *Histochemistry*. 1993; 100:431–440. [PubMed: 7512949]
- Smith K, Graybiel A. A dual operator view of habitual behavior reflecting cortical and striatal dynamics. *Neuron*. 2013; 79:361–374. [PubMed: 23810540]
- Smith KS, Graybiel AM. Habit formation coincides with shifts in reinforcement representations in the sensorimotor striatum. *J Neurophysiol*. 2016; 115:1487–1498. [PubMed: 26740533]
- Surmeier DJ, Plotkin J, Shen W. Dopamine and synaptic plasticity in dorsal striatal circuits controlling action selection. *Curr Opin Neurobiol*. 2009; 19:621–628. [PubMed: 19896832]
- Tecuapetla F, Matias S, Dugue GP, Mainen ZF, Costa RM. Balanced activity in basal ganglia projection pathways is critical for contraversive movements. *Nat Commun*. 2014; 5:4315. [PubMed: 25002180]
- Tepper JM, Tecuapetla F, Koós T, Ibáñez-sandoval O, Kreitzer A. Heterogeneity and diversity of striatal GABAergic interneurons. *Front Neuroanat*. 2010; 4:1–18. [PubMed: 20161990]
- Thorn CA, Atallah H, Howe M, Graybiel AM. Differential dynamics of activity changes in dorsolateral and dorsomedial striatal loops during learning. *Neuron*. 2010; 66:781–795. [PubMed: 20547134]
- West AR, Grace AA. The nitric oxide-guanylyl cyclase signaling pathway modulates membrane activity states and electrophysiological properties of striatal medium spiny neurons recorded in vivo. *J Neurosci*. 2004; 24:1924–1935. [PubMed: 14985433]
- Wickens JR, Begg AJ, Arbuthnott GW. Dopamine reverses the depression of rat corticostriatal synapses which normally follows high-frequency stimulation of cortex In vitro. *Neuroscience*. 1996; 70:1–5. [PubMed: 8848115]
- Yin HH, Mulcare SP, Hilário MRF, Clouse E, Holloway T, Davis MI, Hansson AC, Lovinger DM, Costa RM. Dynamic reorganization of striatal circuits during the acquisition and consolidation of a skill. *Nat Neurosci*. 2009; 12:333–341. [PubMed: 19198605]

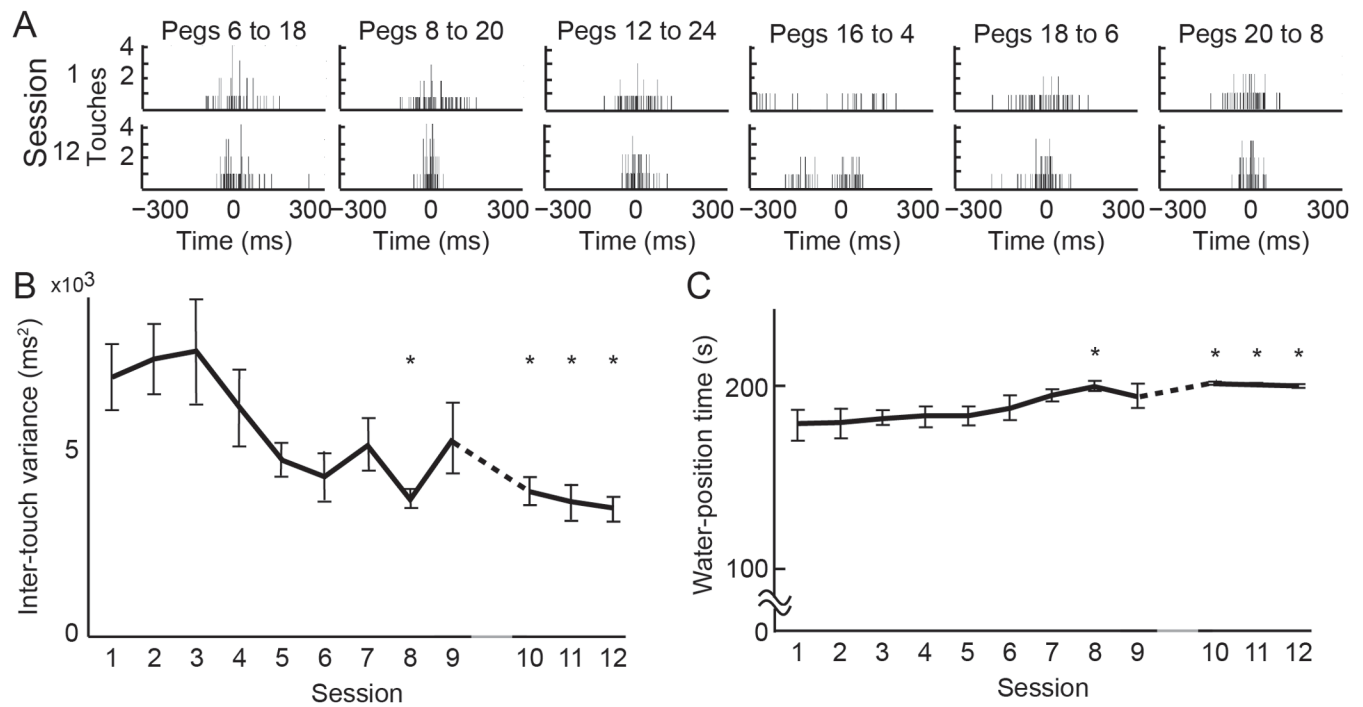
Abbreviations

NMDA	N-methyl-D-aspartate
M1	primary motor cortex
M2	secondary motor cortex
dlSt	dorsolateral striatum

dmSt	dorsomedial striatum
SPN	spiny projection neuron
dSPN	spiny projection neuron of the direct pathway
iSPN	spiny projection neuron of the indirect pathway
Enk	enkephalin
SP	substance P
PV	parvalbumin
nNOS	neuronal nitric oxide synthase
AP5	D-2-amino-5-phosphonopentanoic acid

**Fig 1.**

Step-wheel system. (A) The motor-driven wheel turns at a constant speed. Water reward is delivered from a spout inserted between the right and left pegs. Paw-touch sensor lines attached to every peg (colored line) turned with the wheel and were connected to a slip ring. (B) Five infrared photobeam sensors are equipped for detecting a mouse when running near the spout (a), a mouse at the most downstream of the turn to stop wheel (b), right (c) and left (d) pegs, and wheel turn (e). (C) Three peg-patterns (A, C1 and C2) used in this study.

**Fig 2.**

Performance evaluated by inter-touch variances and water-position times. (A) Touch intervals for pairs of pegs separated by 11 pegs, recorded from a mouse performing pattern C1 in sessions 1 and 12. Histograms aligned to the medians of the touch-intervals. The median is shown as 0, and touch-intervals shorter or longer than the median were shown in left or right to the median, respectively. (B, C) Average inter-touch variances (B) and water-position times (C) across training. A novel peg-pattern (C1) was introduced in session 1, and the mice ($n = 7$) were trained with the pattern for 9 consecutive days (sessions 1 to 9). After a break of 16 days, they were trained again with the same peg-pattern (sessions 10-12). * $p < 0.05$ vs. session 1. Error bars, SEM.

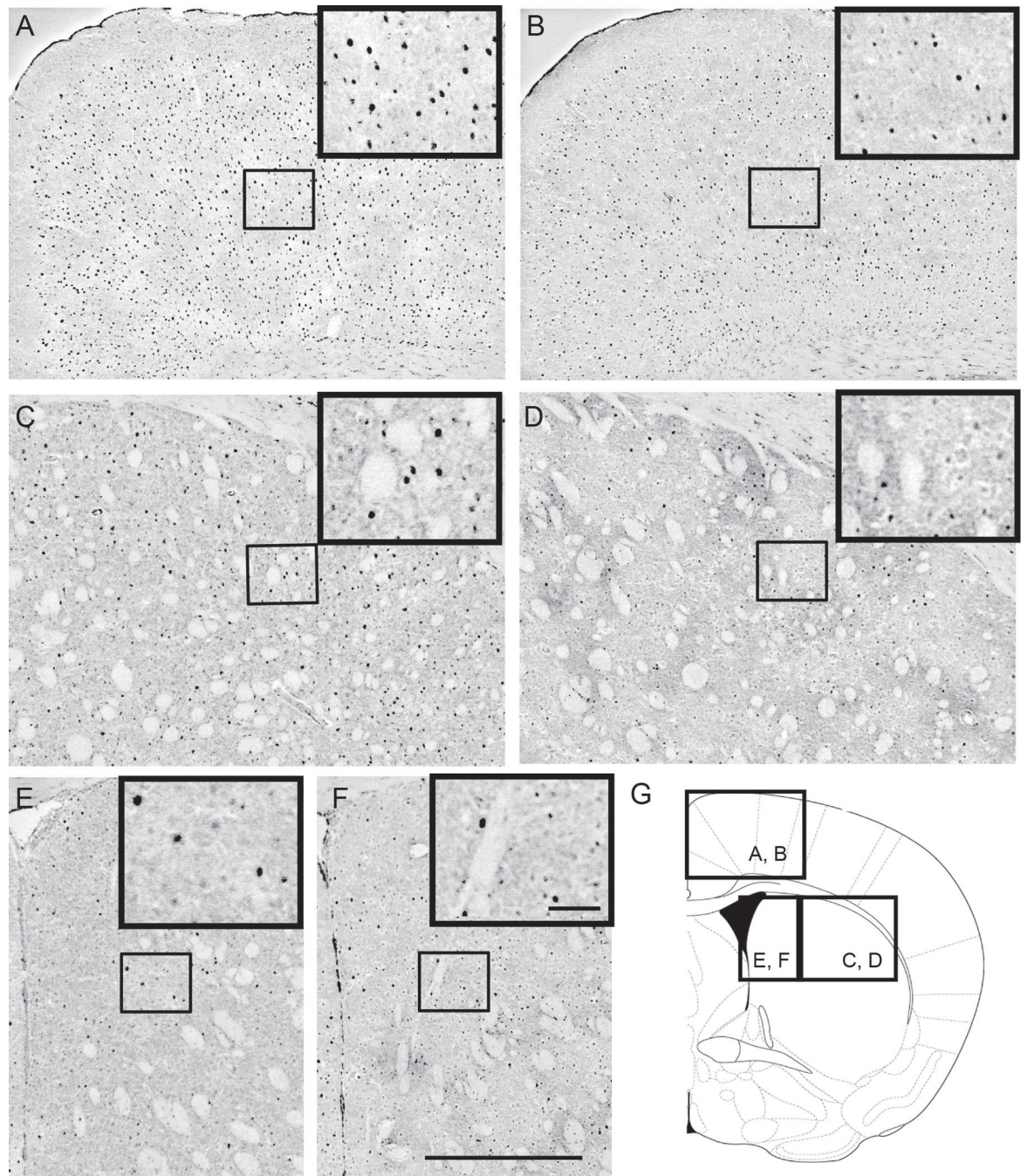
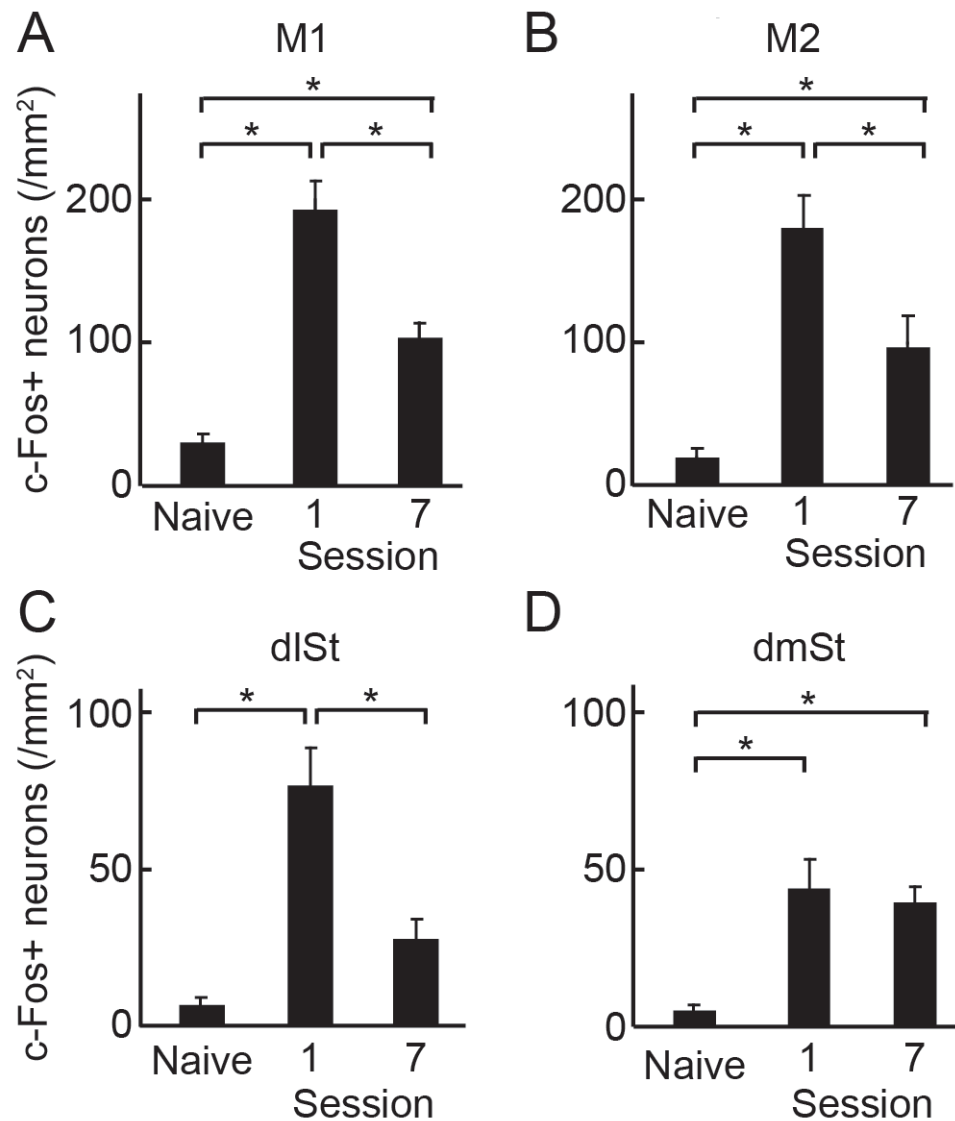


Fig 3. c-Fos expression in the motor cortical areas M1 and M2 (A, B), dorsolateral striatum (C, D) and dorsomedial striatum (E, F), shown for mice trained for one day (A, C, E) or for seven days (B, D, F) on the novel peg-pattern C1. Insets in thick rectangles show enlarged images of regions marked by thin rectangles. Positions of regions shown in A-F are indicated in G (adapted from Paxinos & Franklin, 2001). Scale bar, 500 μ m. Scale bar in insets, 50 μ m.

**Fig 4.**

The numbers of c-Fos⁺ neurons in the M1 (A), M2 (B), dorsolateral striatum (C, dlSt) and dorsomedial striatum (D, dmSt) in home-caged naïve mice and mice trained on peg-pattern C1 in the step-wheel for one or seven days. * $p < 0.05$ (Bonferroni test, $n = 5$). Error bars, SEM.

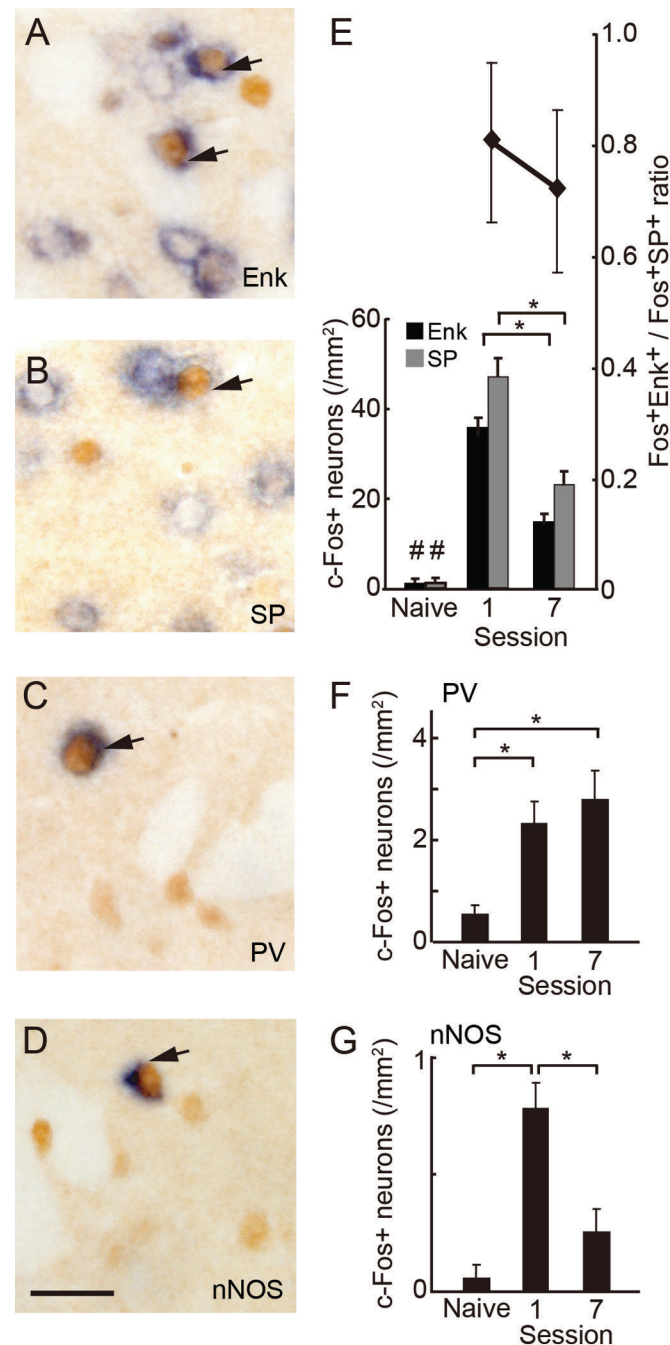
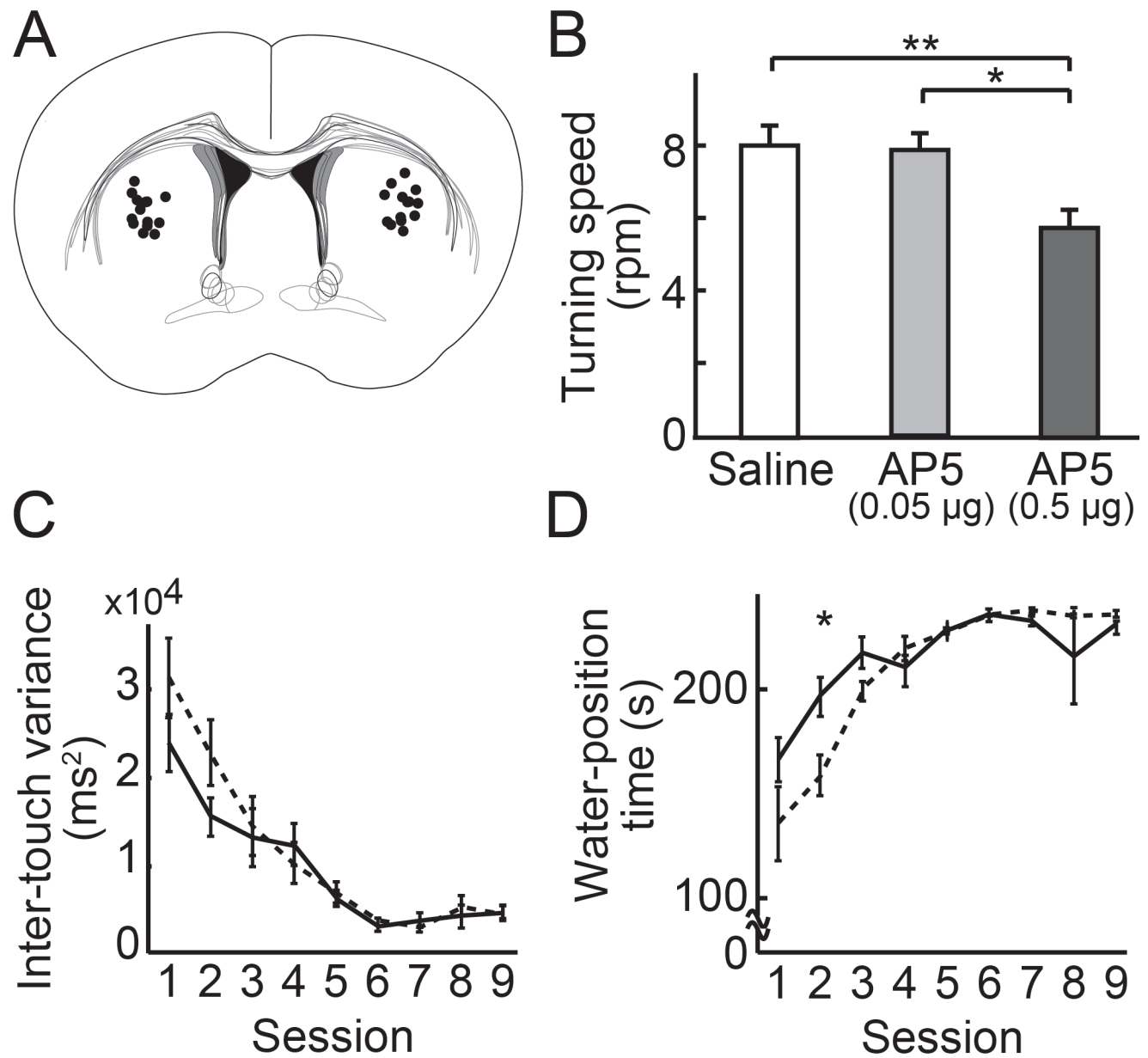
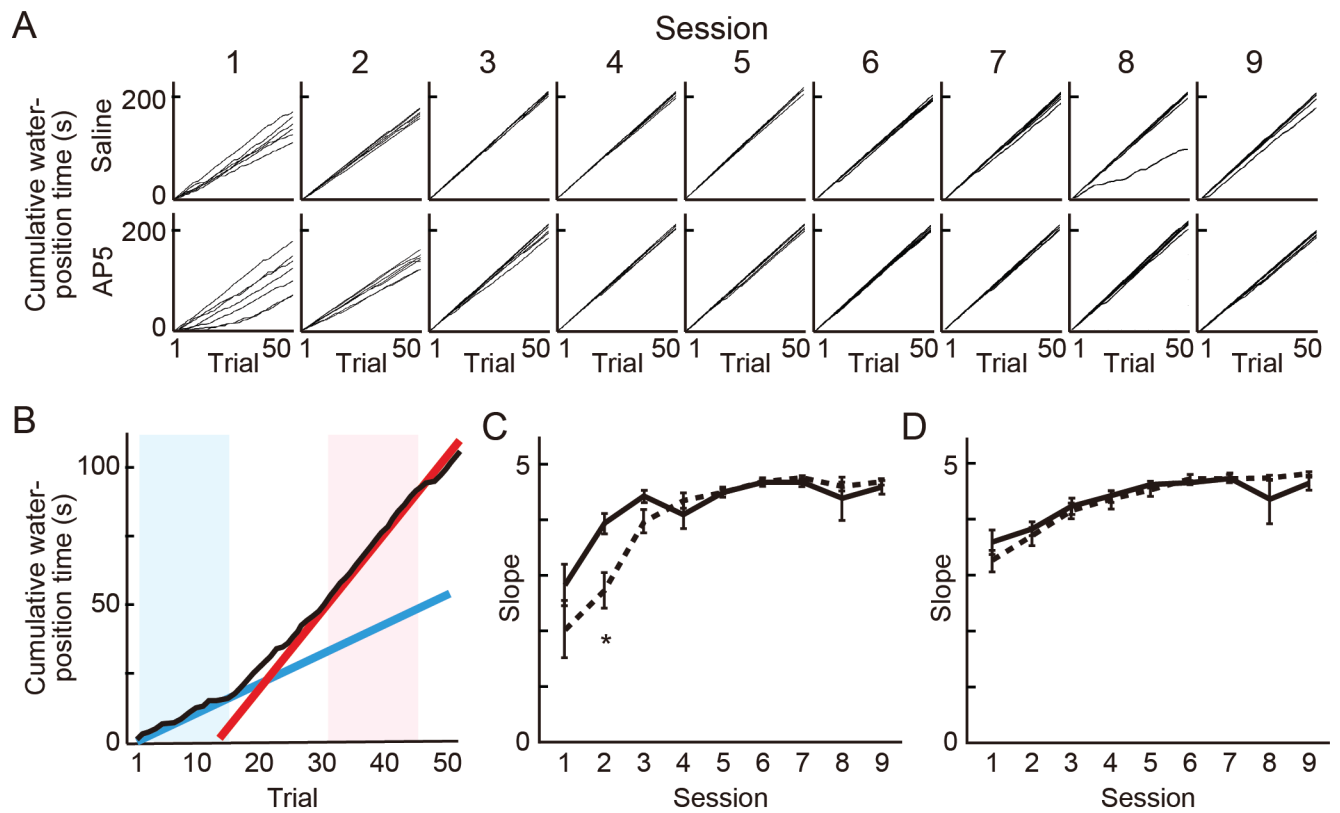


Fig 5. c-Fos expression in striatal projection neurons and interneurons. (A-D) Double staining of c-Fos (brown) and for markers (blue) for dSPNs (A, Enk), iSPNs (B, SP), and interneurons (C, PV; and D, nNOS). Doubly positive neurons are indicated by arrows. (E) The numbers of Fos⁺Enk⁺ (black) and Fos⁺SP⁺ (gray) neurons in the home-caged mice (Naive), and mice trained on a novel peg-pattern C1 for 1 or 7 sessions. The ratio of Fos⁺Enk⁺ SPNs to Fos⁺SP⁺ SPNs is shown as a line graph (right ordinate). $n = 5$ per group. * $p < 0.05$, # $p < 0.05$

0.01 vs. sessions 1 and 7 of the corresponding SPN type. Error bars, SEM. (F, G) The numbers of Fos⁺PV⁺ (F) and Fos⁺nNOS⁺ (G) neurons. Scale bar, 20 mm.

**Fig 6.**

Blockade of NMDA receptor in the dorsolateral striatum. (A) Histological reconstructions of cannula placements in schematic form. (B) Fastest turning speed for control mice injected with saline ($n = 6$), and for mice injected with AP5 at low ($0.05 \mu\text{g}$, $n = 6$) or high ($0.5 \mu\text{g}$, $n = 7$) doses. $*p < 0.05$. $**p < 0.01$. Error bars, SEM. (C, D) Inter-touch variances (C) and water-position times (D) for mice treated with saline (solid line) or AP5 (low dose, dotted line). $*p < 0.05$, two-tailed t test.

**Fig 7.**

Within-session analysis of the water-position time. (A) Cumulative times spent in the water-position in each session for saline-treated (top) and AP5-treated (bottom) mice. (B) Cumulative water-position times over 50 trials (black), with slopes of increment during early (trials 1-15, blue) and late (trials 31-45, red) trials. (C, D) Average slopes for early (C) and late (D) trials for saline-treated (solid line, $n = 7$) and AP5-treated (dotted line, $n = 6$) mice.

* $p < 0.05$, two-tailed t test. Error bars, SEM.