Inhibition of protein synthesis in M1 of monkeys disrupts performance of sequential movements guided by memory

Machiko Ohbayashi[1,2]

[1] Center for the Neural Basis of Cognition, Systems Neuroscience Center, University of Pittsburgh, Pittsburgh, Pennsylvania 15261, USA

[2] Department of Neurobiology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261, USA

*** Corresponding author:**

Machiko Ohbayashi (machiko@pitt.edu)

Abstract

The production of action sequences is a fundamental aspect of human motor skills. To examine whether primary motor cortex (M1) is involved in maintenance of sequential movements, we trained two monkeys (*Cebus apella*) to perform two sequential reaching tasks. In one task, sequential movements were instructed by visual cues, whereas in the other task, movements were generated from memory after extended practice. After the monkey became proficient with performing the tasks, we injected an inhibitor of protein synthesis, anisomycin, into M1 to disrupt information storage in this area. Injection of anisomycin in M1 had a marked effect on the performance of sequential movements that were guided by memory. In contrast, the anisomycin injection did not have a significant effect on the performance of movements guided by vision. These results suggest that M1 of non-human primates is involved in the maintenance of skilled sequential movements.

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Introduction

The ability to perform a sequence of movements is a key component of motor skills, such as typing and playing a musical instrument. How the brain binds elementary movements together into meaningful actions has been a topic of much interest. The preparation for and generation of sequential movements is classically thought to depend on the supplementary motor area (SMA) and the pre-SMA (Roland et al., 1980; Tanji and Shima, 1994; Gerloff et al., 1997; Shima and Tanji, 1998; Nakamura et al., 1998; Hikosaka et al., 2002; Picard and Strick, 2001; Dayan and Cohen, 2011). According to this view, the primary motor cortex (M1) is thought to produce the patterns of muscle activity that are necessary to implement the motor plans

generated by the premotor areas. There is growing evidence, however, that M1 of humans and non-human primates is involved in the acquisition and retention of sequential movements. For example, the activity of some M1 neurons appears to reflect the serial order of potential target stimuli (Pellizzer et al., 1995; Carpenter et al., 1999). Similarly, a large number of M1 neurons (~40%) and metabolic activity reflected aspects of learned movement sequences (Lu and Ashe, 2005; Matsuzaka et al., 2007; Picard et al., 2013). In addition, the functional connectivity of M1 neurons was suggested to be associated with sequential planning of movements (Hatsopoulos et al., 2003). These observations and others imply a more active role for M1 in the acquisition and retention of skilled movement sequences than previously thought (Karni et al., 1995; Dayan and Cohen, 2011). In fact, the results of imaging studies in humans underscore the importance of practice history and skill level for the activation of the motor areas (Krings et al, 2000; Meister et al., 2005).

However, causal experiments to test M1's involvement in the acquisition or maintenance of motor sequences have been challenging. M1 is critical for implementing motor output. Lesion or inactivation of M1 will abolish the motor commands to the spinal cord that generate muscle activity. Instead, we manipulated protein synthesis in M1 to selectively disrupt information storage in this cortical area. This approach has been successful in the experimental dissection and analysis of other memory systems in rodents (Davis and Squire, 1984; Nader et al., 2000; Kleim et al., 2003). In these studies, local injection of an inhibitor for protein synthesis, anisomycin, was shown to interfere with learning and maintenance of memory (Davis and Squire 1984; Nader et al., 2000 a,b; Kandel, 2001; Dudai, 2004, 2012; Kelleher et al. 2004; Rudy 2013; Kleim et al., 2003) .

Results

We trained two monkeys (*Cebus apella*) on two tasks. In one task, the monkeys performed sequential movements guided by memory (Repeating task) (Fig. 1 a, b). As a control task, the monkeys also were trained to perform reaching movements guided by visual cues (Random task, for details see Methods). After the monkeys became proficient with performing the two tasks (> 100 training sessions), we made injections of the protein synthesis inhibitor, anisomycin, in M1. The injections of aniomycin solution (100 mg/ml) were placed at sites in which intracortical stimulation evoked shoulder or elbow movements (Fig. 1c-f, for details see Methods). We analyzed the monkey's behavior before and after the injection separately for each movement in each task. A movement from one target to the next is defined as a trial.

The anisomycin injections had a significant effect on the performance of the movements during the Repeating task (Figs. 2, 3a,b). The injections resulted in a significant increase in the number of errors (Fig. 2, 3; χ^2 test, $p < 0.05$) and a significant decrease in the number of predictive responses, an indication of sequence learning, (Fig. 3c; χ^2 test, $p < 0.05$) during the Repeating task. In contrast, performance of visually guided movements during the Random task was not significantly disrupted (χ^2 test, $p > 0.05$).

The incorrect responses during the Repeating task can be categorized as two types: errors of accuracy and errors in direction. An accuracy error is a reach performed in the correct direction (e.g., to the right in move 1-5), but to an endpoint outside of the correct target (e.g., short of target 5). As shown in Fig. 2a, before the injection, the monkey made correct contact to target 5 on 96% of the trials in the Repeating task (sequence 5-3-1). After the anisomycin injection,

the number of incorrect responses increased dramatically. The monkey then made accuracy errors on 45% of the trials (Fig. 2a).

A direction error was considered to be a reach performed in the direction opposite to the correct target (Fig. 2b, E_D). For example, movement 2-4 requires a rightward movement from target 2 to target 4. As shown in Fig. 2b, the monkey made a correct movement to target 4 on 93% of the trials in the Repeating task (sequence 1-2-4) before the injection. After the anisomycin injection, the animal moved his arm to the left from target 2 and made direction errors on 18% of the trials in the Repeating task (Fig. 2b). This type of error suggests a deficit in selecting the movement component in the sequence. For the injection session illustrated in Figs. 2 and 3, the error rate increased significantly for five of the six movements performed during the Repeating task.

Overall, we observed a significant increase in error rate for all of six injection sessions (χ^2 test, $p < 0.05$; monkey N, n = 4; monkey R, n = 2). The effect of anisomycin was more pronounced for certain moves of the learned sequences (Figs. 3, 4). The affected moves varied between sessions. The error rate increased by an average of 42% for the most affected movements during the Repeating task (Fig. 4b; paired t-test; $p = 0.002$).

As noted above, the anisomycin injections also caused a change in the number of predictive movements during the Repeating task. We defined predictive responses as movements completed before the presentation of the next target cue or in less than 150 ms after the presentation of the next target cue (see Methods). Before the anisomycin injection in monkey N, session No. 2, the animal made predictive responses on > 90% of the trials (Fig. 3c). After

the anisomycin injection, the number of predictive movements decreased dramatically. For movements from target 5 to target 3 in sequence 5-3-1, the number of predictive responses decreased by 55% of the total trials (Fig. 3c, χ^2 test, $p < 0.001$).

Overall, we observed a significant decrease in the number of predictive movements for all of six injection sessions (χ^2 test, $p < 0.05$). The number of predictive responses decreased an average of 56 % for the most affected movements during the Repeating task (Fig. 4c; paired t-test, $p < 0.001$). A decrease in the number of predictive responses suggests an increase in the time for movement selection.

As control experiments, we inactivated M1 by injecting muscimol, a GABA agonist, into the shoulder representation of M1 in two sessions. We examined the performance of the animal on the Random and Repeating tasks as we did for anisomycin injections into M1. We found that M1 inactivations impaired performance on both the Random and Repeating tasks ($\chi 2$ test, $p < 0.05$). The results indicate that M1 inactivation caused an indiscriminate deficit of motor production. Injection of saline into M1 did not have any significant effects on performance of both the Random and Repeating tasks ($\chi 2$ test, $p < 0.05$).

Discussion

In the present study, localized inhibition of protein synthesis in M1 resulted in a selective deficit in the performance of internally generated sequential movements during the Repeating task. This observation emphasizes the importance of M1 for the generation of sequential movements that are memory guided. Our results suggest that, although M1 is critical for movement production, it also is involved in the maintenance of skilled sequential movements.

The acquisition and maintenance of long-term memory requires a series of cellular and molecular changes that involve translation (i.e. protein synthesis) (Davis and Squire, 1984). The role of protein synthesis in learning and memory has been intensively studied in rodents in the context of fear conditioning (Davis and Squire 1984; Nader et al., 2003; Kandel, 2001; Dudai, 2004, 2012; Kelleher et al. 2004; Rudy 2008; Ryan et al., 2015). Similarly, an injection of a protein synthesis inhibitor, anisomycin, into rat M1 severely disrupted the maintenance (Kleim et al., 2003) or the learning (Luft et al., 2004) of a skilled forelimb reaching task. The injection of anisomycin also caused a reduction in synapse density and in the thickness of the post synaptic density in M1 *in vivo* (Kleim et al., 2003). Studies using brain slices showed that protein synthesis is required for long-lasting synaptic plasticity (late long-term potentiation) (reviewed in Kelleher et al. 2004) and spine-head enlargement and growth during learning (Steward and Levy, 1982; Steward and Fass, 1983; Steward and Schuman, 2003; Tanaka et al., 2008). Several observations indicate that the effects of protein synthesis inhibitors on long-lasting synaptic plasticity are likely to be a specific consequence of their translational blockade (Linden, 1996; Huber et al. 2000; Beaumont et al. 2001; Kelleher III et al., 2004; Sadowski et al., 2011). These studies in rodents support our finding that inhibition of protein synthesis in M1 of monkeys interfered with performance of sequential movements guided by memory.

M1 is densely interconnected with the dorsal premotor cortex proper (PMd) (Dum and Strick, 2005). We recently demonstrated that inactivation of the PMd resulted in a selective deficit in the performance of internally generated sequences (Ohbayashi et al., 2016). We proposed that the PMd contributes to the internal generation of sequential movements through maintaining motor-motor associations. Our current results are consistent with the PMd functioning as a

major source of input to M1 to guide the performance of internally generated sequences. Previous studies reported that extended practice on a sequence of movements resulted in dramatic alterations in the functional activation and neural responses of M1 (Matsuzaka et al., 2007; Picard et al., 2013). In addition, human imaging studies found a functional and structural change in M1 of professional musicians (Elbert et al., 1995; Amunts, K et at., 1997; Gaser and Dchlaug, 2003; Schwenkreis et al., 2007; Draganski and May, 2008; Herholz and Zattore, 2012; Zatorre et al., 2012; Sampaio-Baptista and Johansen-Berg, 2017; Wenger et al., 2017). These studies, along with our current results, suggest that M1 is involved in the maintenance of sequential movements after extensive practice. M1's specific contribution to the acquisition and retention of sequential movements needs to be further explored in future experiments.

Materials and Methods

The care of the monkeys and the experimental protocols adhered to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals. All procedures used followed institutional guidelines and were approved by the Institutional Animal Care and Use Committee.

Behavioral task. Two monkeys (*Cebus apella*, two males weighing 3.2 kg and 3.7 kg) were trained to perform two tasks that required sequential reaching movements to targets on a touch sensitive monitor with their right arm (Fig. 1a, b) (Matsuzaka et al., 2007; Picard et al., 2013; Ohbayashi et al., 2016). In the Random task, the reaching movements were guided by visual targets displayed on a touch screen monitor. Each new target was presented according to a pseudorandom sequence. Contact of the correct target triggered the display of the next target

after 100 ms delay. After the monkeys became proficient in the performance of the Random task (~ 50 days of practice), they were introduced to the Repeating task. In the Repeating task, new targets were presented according to a three-element repeating sequence. We trained animals on repeating sequences: “5-3-1-5-3-1 . . .” and “1-2-4-1-2-4 . . .” or “2-3-4-2-3-4 ...” (Fig. 1b, two sequences for monkey N, one sequence for monkey R). New targets during the Repeating task were presented 400 ms after contact of the preceding target. This delay promoted the performance of predictive responses during the Repeating task in which the animal moved to the next target in a sequence before the target was presented. Each task was performed continuously in blocks of 200–500 trials. A liquid reward was given after every four to five correct responses. Both monkeys became proficient with performing the two tasks after > 50 training sessions. The injection experiments were performed after a monkey had more than 100 training sessions on each sequence.

Surgery. We implanted a head restraint device, along with an MR compatible chamber for micro-injection, on an animal's skull using small screws and dental acrylic. All surgical procedures were performed under general anesthesia (1-3% isofluorane) using aseptic techniques. The animal received antibiotics and analgesics after surgical procedures. The chamber’s placement over M1 was verified using structural MR images taken prior to and after the surgery (Fig 1d). When task performance returned to the pre-surgical level, we performed a craniotomy to expose the cortex in the chamber.

Intracortical microstimulation. We used intracortical microstimulation to identify the arm representation in M1 and to physiologically define the border between M1 and the PMd (Dum and Strick, 2005 and Ohbayashi et al., 2016). We used glass-coated Elgiloy microelectrodes

(0.6–1.5 MΩ at 1 kHz) to deliver intracortical microstimuli. A constant-current stimulator was used to deliver cathodal pulses (1- 40 μA intensity, 10-20 cathodal pulses, 333 Hz, 0.2 ms duration) at a depth of 1500 μm below the cortical surface (Dum and Strick, 2005; Ohbayashi et al, 2016). Stimulus intensity was measured with a current monitor (Ion Physics). The motor response evoked by stimulation was determined by visual observation and muscle palpation. The response threshold was defined as the lowest stimulus intensity necessary to evoke a response on ~80% of the trials.

Injections of pharmacological agents. We injected anisomycin (an inhibitor for protein synthesis), muscimol solution or sterile saline at 1.5 mm below the cortical surface using a 30 gauge cannula connected to a 10 μl Hamilton syringe (0.2 μl every 30–60 s). We prepared solutions of anisomycin (100 μg/μl in ACSF, pH 7.2-7.4) and muscimol (5 mg/ml in saline) from commercially available powders (Sigma-Aldrich, MO). The cortical sites of anisomycin injection in monkey N are displayed in Figure 1e (injection session No. 2). For each injection session, we injected total of 5 or 10 μl anisomycin solution into M1 (5μl, n=5; 10μl, n=1). To cover a large portion of the arm representation in M1, the anisomycin solution was injected at two to four sites in the arm representation area of M1 (Fig. 1f, 1-3 μl at each site). The cannula was left in place for ~5 min to allow diffusion of the solution and prevent its reflux and then removed. The effect on performance of the tasks was tested 20-24 hours after the injection of an inhibitor of protein synthesis. We did experiments with anisomycin injections 4 times in monkey N and 2 times in monkey R. Injections were spaced at least 7 days apart. In separate experiments, we also injected 1-3 μl of muscimol to inactivate M1 function to compare the results with anisomycin injections. The effect of muscimol on task performance was tested 20 min after the injection to allow for some diffusion of the chemical in the brain tissue before resuming the task.

Saline (5 μl) was injected as a control in separate experiments. During a post-injection test session, blocks of Random and Repeating trials were alternated at frequent intervals to sample the animal's performance evenly as the effect of the test substance emerged and intensified. Measurement of performance on days prior to an injection and/or on trial blocks preceding an injection was used as the baseline for comparison with post-injection performance.

Analysis of performance. For every trial of each task, we recorded various task parameters and measures of performance. The effect of an injection was assessed by examining the following: changes in the percentage of correct responses, types of incorrect responses, changes in Movement Time (MT) and Response Time (RT), percentage of predictive responses (defined as RT < 150ms) and contact points on the touch screen (Ohbayashi et al., 2016). We defined MT as the interval between the release of contact from one target to touch of the next target. We defined RT during the Random task as the time between the presentation of a new target and contact of that target minus 100 ms delay time. We defined RT during the Repeating task as the time between contact of two targets in a sequence minus 400 ms delay time. A negative RT indicates that the animal is internally generating the response before the visual cue is presented. After ~50 days of training on the first Repeating sequence, the monkey made predictive responses (RT < 150 ms) without using visual cues in more than 80% of the trials. When RT was less than 150 ms the trial was considered to be a predictive response. We excluded the following trials from analysis: 1) trials after errors because in this case the trial was repeated; 2) trials during the Random task with an RT less than 150 ms because the monkey may not have been paying attention to the task, tried to guess the next target or have attempted to perform a move of a Repeating sequence. For each movement, we used χ^2 tests with Holm–Bonferroni's correction to examine the significance of changes in error rate and

predictive responses. We used t-tests with Holm–Bonferroni's correction to examine changes of MT and RT. The effect of anisomycin was more pronounced for certain moves of the learned sequences. For each injection session, a movement of the Repeating task that showed the largest and weakest change of error rate were identified. The data of these movements during the Repeating task and the data of corresponding movements during the Random task were used for population analysis, average and SE of error rates and percentage of predictive responses, in Figure 4.

Author contributions: M.O. designed research, performed research, analyzed data and wrote the paper.

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Competing interests

The authors declare no competing financial interests.

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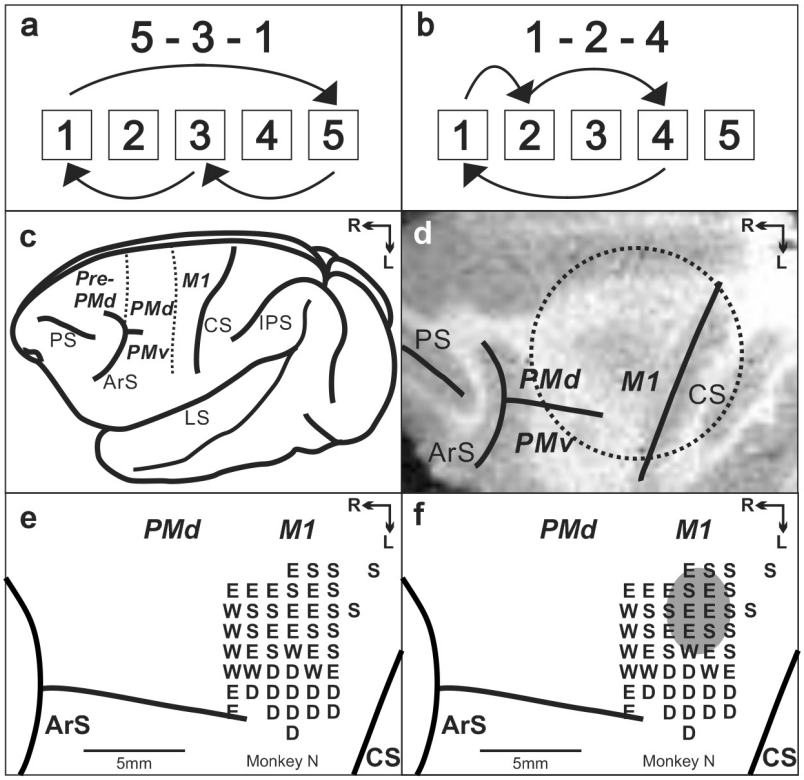

Figure 1: Task and cortical maps for monkey N.
a, b. Sequences in the Repeating task. c. Lateral view of cebus brain. Dashed lines indicate the M1-PMd border and the pre-PMd-PMd border. ArS: arcuate sulcus; CS: central sulcus; IPS: intra parietal sulcus; LS: lateral sulcus; pre-PMd: pre-dorsal premotor cortex; PMd: dorsal premotor cortex; M1: primary motor cortex. R: rostral; L: lateral. d. MRI image after the chamber implantation for monkey N. The dotted circle indicates the chamber outline. e. Intracortical stimulation map from monkey N. Letter indicate the movements evoked at each site. S:Shoulder; E: Elbow; W: Wrist; D: Digit. f. Anisomycin solution was injected in the area indicated by a gray oval.

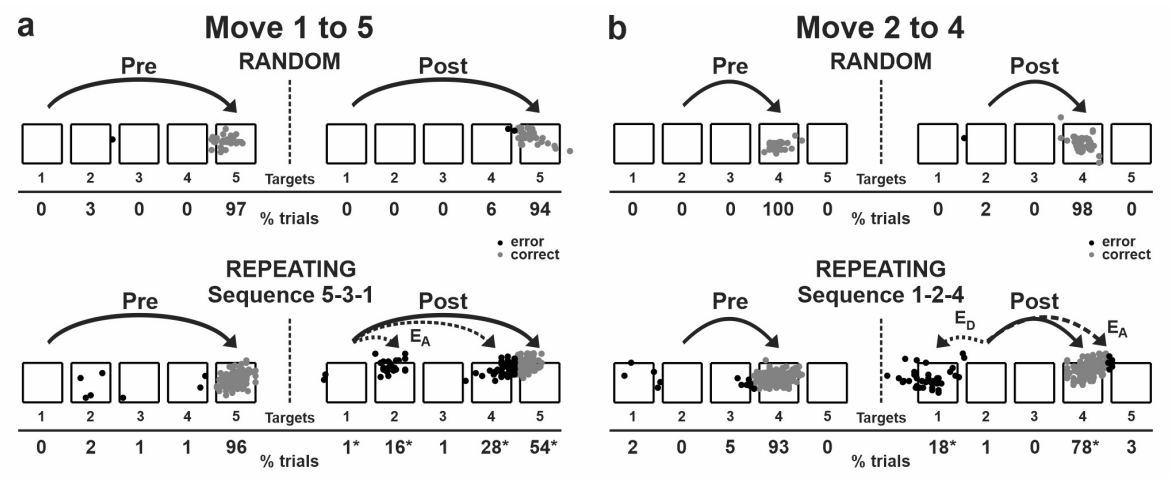

Figure 2: Reaching end points before and after anisomycin injection.
Left: pre-injection; right: post-injection. E_A: Accuracy errors; E_D: Direction errors; gray dots: correct response; black dots: error response. The anisomycin injection was placed at sites shown in Fig. 1f in monkey N. **a**, Reaching end points for movement from target 1 to target 5. The monkey was performing sequence 5-3-1 during the Repeating task. **b**, Reaching end points for movement from target 2 to target 4. The monkey was performing sequence 1-2-4 during the Repeating task. Percentage of trials ending in each target are given below the targets. Touches between targets were counted as touches to the closest target. *$p < 0.05$.

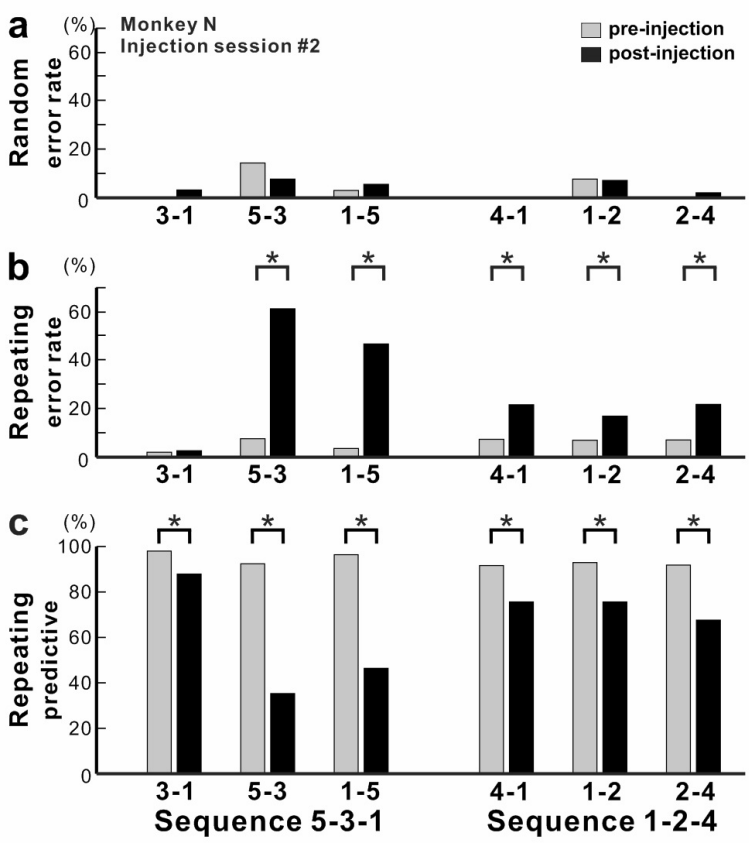

Figure 3: Effect of anisomycin injection on performance of the task.
a– c, The performance data for an injection session #2 in monkey N (Fig. 1f, Fig. 2). a, Error rate in the Random task. Anisomycin injection did not have any effects on number of errors during the Random task (χ^2 test, p = 0.385 for move 3-1, df=1; p = 0.639 for move 5-3, df=1; p = 0.624 for move 1-5, df = 1; not significant for move 4-1, df=1; p = 0.957 for move 1-2, df=1; p = 0.371 for move 2-4, df=1). b, Error rate in the Repeating task. After the anisomycin injection, the number of errors increased dramatically in 5 of 6 movements in the Repeating task (χ^2 test, $p < 0.001$ for moves 5-3, 1-5, 4-1 and 2-4, df=1; p=0.689 for move 3-1, df=1; p=0.004 for move 1-2, df = 1). c, Predictive responses. The percentage of predictive responses decreased significantly after the anisomycin injection (χ^2 test, $p < 0.001$ for all movements, df = 1).

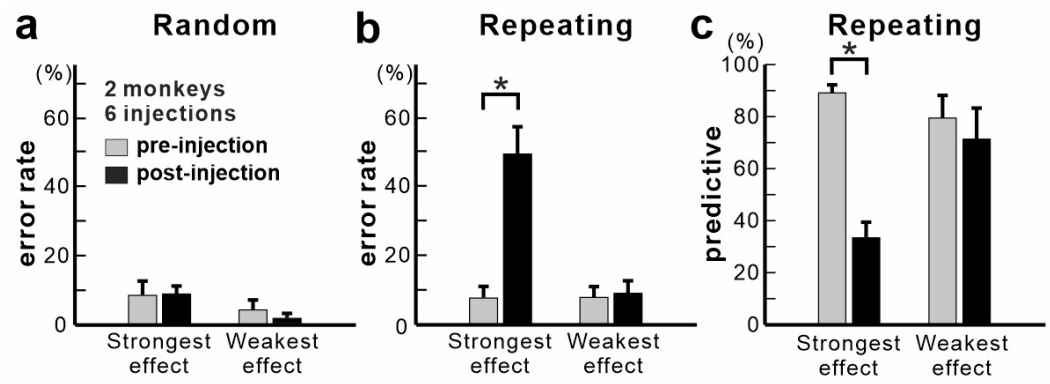

Figure 4: Population data for anisomycin injections.
a, Average error rate in the Random task. Anisomycin injections did not have an effect on the number of error responses in the Random task (paired t-test, 6 injection experiments, $p = 0.901$ for strongest effect, df=5; $p = 0.274$ for weakest effect, df = 5). b, Average error rate in the Repeating task. The error rate in the Repeating task increased significantly after the anisomycin injections (paired t-test, 6 injection experiments, $p = 0.002$ for strongest effect, df=5; $p = 0.497$ for weakest effect, df = 5). c, Average predictive responses in the Repeating task. The percentage of predictive responses decreased after anisomycin injections in M1 (paired t-test, $p < 0.001$ for strongest effect, df=5; $p = 0.419$ for weakest effect, df = 5). *$p < 0.05$.