Title: Gradual consolidation of skilled sequential movements in primary motor cortex of non-human primates

Authors: Machiko Ohbayashi[1,2*], Nathalie Picard[1,2]

Affiliations:

1. Department of Neurobiology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261, USA.
2. Systems Neuroscience Center, University of Pittsburgh, Pittsburgh, Pennsylvania 15261, USA.

Corresponding author: Machiko Ohbayashi (machiko@pitt.edu)

Abstract

Expert-level performance of sequential movements can be achieved through extensive practice. The primary motor cortex (M1) is suggested to play a key role in acquiring and retaining sequential movements, with evidence of reorganization in M1 following prolonged practice, such as changes in fMRI activation in humans and altered neuron activity in monkeys. Here, we examined the timeline of plastic changes in M1 of monkeys during learning of sequential movements. A challenge in studying a role of M1 in learning is that its inactivation impairs movement, masking learning processes. To address this, we used a protein synthesis inhibitor to disrupt memory consolidation in M1 during learning. Our results show that inhibiting protein synthesis in M1 disrupted memory-guided performance at all stages of learning, though the effect decreased with continued practice. This suggests that neural traces for sequential movements are repeatedly consolidated through protein synthesis, with the rate of consolidation slowing as learning progresses.

Teaser

The neural traces for skilled sequential movements are repetitively consolidated through protein synthesis in the primary motor cortex of monkeys.

Introduction

Many daily activities, such as playing a musical instrument and typing, rely on attaining a high level of proficiency in performing sequential movements. The sequential movements can be acquired and improved to an expert level through extensive practice, eventually becoming an established motor skill. Once the structure of a sequence is learned, continued daily practice over an extended period allows for slow improvement in speed and accuracy, key indicators of motor skill learning. This slow incremental learning is considered a fundamental aspect of motor skill development.

Studies in humans and non-human primates have shown that M1 activity changes during the early stage of sequence learning. In humans, the changes in M1 activity measured with fMRI occur with short-term training of sequential movements (1-17). In monkeys, neural activity in M1 is modulated by the sequence components during the early stage of learning (18, 19).

Changes of M1 after the extensive practice of sequential movements are also reported in human and non-human primate studies. In humans, years of extensive practice have been proposed to modify the structure and functional activation of M1 (16, 20-25). The functional activation of M1 during the performance of sequential tasks was reduced or more focused in professional musicians compared to non-musicians or amateurs (20-24). The reduced activation is considered evidence of increased efficiency of the motor system, or the need for a smaller number of active neurons to perform a highly trained set of sequential movements.

The volume of M1 has been reported to be larger in professional musicians compared to amateurs or non-musicians (26-33). The structural changes have been proposed to be supported by processes occurring at the synaptic level, including intracortical remodeling of dendritic spines and axonal terminals, glial hypertrophy, and synaptogenesis as a result of extensive practice (29-31, 34). Similarly, the plasticity of the white matter structure of M1 was correlated with the amount of time devoted to practice, suggesting that myelination may be increased by neural activity in fiber tracts during training (35, 36).

In non-human primates, after extensive training, M1 neurons were differentially active during the performance of visually guided and internally generated sequential reaching (37, 38); the uptake of 2DG (a measure associated with presynaptic activity) was low in monkeys that performed highly practiced, internally generated sequences of movements compared with monkeys that performed visually guided reaching (38). The change of fMRI activation in the M1 of humans, decreased 2DG signal in the M1 of non-human primates, and the enlarged volume of the M1 in musicians may all reflect the reorganization in M1 with extensive practice.

These diverse findings converge to the concept that M1 is involved in learning and maintenance of motor skills such as sequential movements through practice. While these studies suggest that activity patterns in M1 change in the early phase of learning and as a result of extensive practice, they do not conclusively indicate when and how the plasticity occurs in M1 during learning. It remains unclear whether M1 undergoes changes throughout all phases of learning or only during specific stages. Additionally, the relationship between the slow improvement of sequence performance and the functional and structural changes in M1 is not yet

fully understood. A traditional approach to this problem would be lesion or inactivation studies. However, in the case of M1, it has been challenging to causally examine when and how plastic changes associated with long-term learning occur in M1 because M1 is critical for implementing motor output. A lesion or inactivation of M1 will abolish the motor commands to the spinal cord that generate muscle activity, so that it would be impossible to evaluate M1's involvement in learning. Therefore, we targeted information storage in M1 instead of motor output. We injected an inhibitor for protein synthesis in M1 to interfere with information storage and, consequently, learning.

Inhibitors for protein synthesis have been widely used in the experimental dissection and analysis of other memory systems in rodents, like fear conditioning. Local injection of an inhibitor of protein synthesis (e.g., anisomycin) into amygdala, hippocampus or motor cortex of rodents disrupted the learning, maintenance or reconsolidation of memory (39-51). These studies indicated that protein synthesis inhibition can interfere with memory formation. We applied this approach to M1 of monkeys and found that protein synthesis inhibitor successfully interfered with the well-trained (~100 days) sequential movements (52). Here, we applied this approach to examine when and how M1 contributes to learning of sequential movements during repetitive practice over extensive time in non-human primates.

Results

Learning of sequential reaching task

We trained two monkeys (Cebus apella) on two types of reaching tasks (Random and Repeating tasks, Fig. 1, for details see Materials and methods). In the Repeating task, we presented visual instruction cues according to a repeating sequence of 3 elements (e.g., 5-3-1-5-3-1- ..., Fig. 1A, B) 400 msec after monkey's contact of the preceding target. If the monkey made a correct response during the 400 msec delay, the instruction cue was not presented, and the task proceeded to the next target in the sequence. With practice, monkeys performed the correct sequence in the Repeating task using predictive responses instead of using visual instructions to guide their reaching (Fig 1 C, D). Thus, the monkeys performed memory-guided sequential movements during the Repeating task. As a control task, we trained the monkeys to perform the Radom task in which the visual instruction cues were presented in pseudo random order. Thus, the monkeys performed reaching movements guided by visual cues (Random task). The two tasks were performed continuously in alternating blocks of 200-500 trials in each daily session. The monkeys predicted the targets in a Repeating sequence in more than 80% of trials after 50 days of training (Fig. 1C).

We trained monkeys to perform two to three sequences of the Repeating task, with the introduction of new sequences staggered over time. We introduced a new sequence more than 50 days after the introduction of the previous one (Fig. 1D; see Methods). The monkey performed each sequence and the Random task in alternating blocks during each training session. Therefore, the training duration of each sequence differed at the time of experiments. For example, in Fig. 1D, at day 393, training duration was 357 days for the 1st sequence, 151 days for the 2nd

sequence and 42 days for the 3rd sequence. As shown in Figure 1, the Response Time for each sequence decreased independently as training proceeded, which provided time points when the sequences were in a different learning phase. The staggered training design enabled us to examine whether the effect of pharmacological injection depends on the training duration and learning phase by comparing the effect between sequences in a single injection session in addition to a comparison between injection sessions.

The inhibition of protein synthesis selectively interfered with the performance of repeating sequences guided by memory, while sparing visually-guided reaching.

When the monkey was able to perform the Random task and more than two sequences of the Repeating task, we made micro injections of the protein synthesis inhibitor, anisomycin (100 mg/ml), at sites in M1 where intracortical microstimulation evoked shoulder or elbow movements (Figure 2, see Methods). For each session, we analyzed the monkeys' behavior before and after the injection separately for each movement in the Random task and the Repeating task.

During the injection session shown in Figure 3, monkey N performed three sequences, each with different training durations: short for sequence '2-3-4' (Fig. 3 A-C), long for sequence '1-2-4' (Fig. 3 D-F) and extensive for sequence '5-3-1' (Fig. 3 G-I). The injection of anisomycin in M1 had a significant effect on performance of all the sequences of the Repeating task. The effect of anisomycin was more pronounced for certain moves of the learned sequences. Figure 3 shows data of the most affected movement of each sequence. The injections resulted in a significant increase in the number of incorrect responses (Fig. 3 B, E, H, $\chi 2$ test, $p<0.05$) and a significant decrease in the number of predictive responses, an indication of sequence learning, (Figure 3 C, F, I; $\chi 2$ test, $p<0.05$) for all three sequences of the Repeating task. In contrast, performance of visually guided movements during the Random task was not significantly disrupted ($\chi 2$ test, $p>0.05$).

The monkey made two types of incorrect responses during the Repeating task: errors of accuracy and errors in direction. An accuracy error is a reach performed in the correct direction, but to an endpoint outside of the correct target. As shown in Figure 3D (sequence 1-2-4), after the anisomycin injection, the animal moved his arm to the left from target "4", the correct direction, but the moves fell short in 50% of trials (Fig. 3D, bottom row). A direction error is a reach performed in the direction opposite to the correct target. For example, movement "3–4" requires a rightward movement from target "3" to "4". As shown in Figure 3A (sequence 2-3-4), after the anisomycin injection, the animal moved his arm to the left from target "3" and made direction errors on 57% of the trials during the Repeating task (Fig 3A, bottom row). The direction errors suggest a deficit in selecting the proper movement component and transitioning from one movement to the next. Given the increase of direction errors and unaffected performance in the Random task, we attribute the performance changes observed in the Repeating task to the injection's effect on memory.

After the anisomycin injection, the Response Time in the Repeating task of the affected movements increased. We categorized the trials with longer RT (RT > 150 msec) as non-

predictive responses (see Methods). We used the percentage of predictive trials as another indicator to assess the effect of injections on task performance (Fig. 3C, F, I). The percentage of predictive trials decreased from 92.4% to 35.3% for the most affected movement, from target 5 to 3 in Fig. 3, during the Repeating task ($\chi 2$ test, $p<0.001$). In the Repeating task trials with longer RT, the monkey paused the arm movement, redirected the arm and made a correct response within 800 msec after the presentation of the visual cue. This behavior suggests that the monkey used the visual cue information to redirect his aim and make a correct response, which was possible because the injection did not affect the performance of visually-guided movements (Random task). Therefore, the increase of RT after the anisomycin injection also suggests that the injection disrupted the memory guided sequential movements.

Notably, the effect of anisomycin injection on error rate of the 'Short-term' trained sequence was larger than its effect on the 'Long-term trained' and 'Extensively' trained sequences (Fig. 3, 4) (*t-test*, $p<0.05$; Fig. 4A). Similarly, the decrease of the percentage of predictive trials and the increase of the Response Time (RT) of the 'Short-term' trained sequence were significantly larger than that of the 'Extensively trained' sequence (Fig. 4B and C, see Methods for the definition of predictive trials and RT). This suggests that the neural representation of 'Long-term' and 'Extensively' trained sequence is less vulnerable to interference.

The performance of Repeating sequences recovered after a few days of training.

After the injection, we continued daily training of the monkeys and monitored changes in their performance. Figure 4D-L shows the performance of the Repeating task from 3 days before to 6 days after the injection session shown in Fig 3. Following the anisomycin injection into M1, the number of correct responses (Fig. 4D, G and J) and the percentage of predictive trials (Fig. 4 E, H and K) decreased across all three sequences of the Repeating task. Additionally, the average Response Time (RT) increased for all three sequences (Fig. 4F, I and L). The monkey's task performance recovered to the baseline levels after 2-3 days of training for all sequences of the Repeating task (Fig. 4) ($\chi 2$ test, $p<0.05$). There was no significant difference in the recovery periods between sequences with varying training durations. The performance recovery time following the protein synthesis inhibition was consistently short, 2-3 days (48-72 hours), regardless of training duration. This recovery period aligns with rodent studies showing that anisomycin injection inhibited protein synthesis in the injected brain area for 48 hours (45). These findings suggest that protein synthesis inhibition disrupted memory traces around the injection site.

The performance deficits in the memory-guided sequential movements observed after anisomycin injection was specifically due to the inhibition of protein synthesis, rather than nonspecific neural dysfunction.

Although anisomycin has been widely used in rodents to study memory systems, our studies are the first to apply it to investigate memory in non-human primates (52). We verified that the observed behavioral effects (Fig. 3) were specifically due to protein synthesis inhibition affecting memory, using three different approaches. First, we inactivated M1 by injecting

muscimol, a $GABA_A$ agonist, into the shoulder representation area of M1 (Fig. 5 A-C). The inactivation resulted in a significant increase in the number of incorrect responses both in the Random and Repeating tasks (Fig. 5 B, C: $\chi 2$ test, $p<0.05$). In both tasks, the monkey's reach fell short of the correct target after the muscimol injection (accuracy errors, 41% for Random; 32% for Repeating) (Fig. 5 A, B). The number of direction errors did not significantly increase after the injection in both tasks (Repeating, pre-injection: 4%; post-injection 6%). Contrary to the effects of anisomycin, the effects of muscimol injections on task performance was primarily on the accurate movement production in both the Random and the Repeating tasks. The difference suggests that the deficit in task performance after the anisomycin injection was likely to be a specific consequence of the induced translational blockade, rather than any nonspecific inhibition or dysfunction of neural activity in M1.

Second, to validate the notion that the anisomycin effect is due to its inhibition of protein synthesis, we injected another type of protein synthesis inhibitor, cycloheximide (44). Anisomycin and cycloheximide have distinct mechanisms of action and different side effects (44). The injection of cycloheximide in M1 resulted in a significant increase of incorrect responses during the Repeating task but did not have an effect during the Random task. During the Repeating task, the error rate increased from 3.6 % to 42.5% for the movement “1-2” for the sequence ‘1-2-4’ ($\chi 2$ test, $p<0.05$). On the other hand, the error rate of the movement ‘1-2’ performed during the Random task did not increase at all (Pre: 0%; Post: 0%). The consistency of the results with the two pharmacological agents suggests that the effect on behavior is due to the common effect of the agents, inhibition of protein synthesis.

Third, we recorded the activity of neurons before and after the anisomycin injections and evaluated the effect of injection on neural activity (Fig. 5 D-F). Fig. 5D shows activity of two M1 neurons during the Random task before and after injections. The neurons were recorded 1 mm away from the injection site. We analyzed neural data collected during the Radom task because the anisomycin injection caused a large increase in number of errors during the Repeating task, as we showed previously (Fig. 3), which prevented the fair comparison of neuron activity during similar movements. Both neurons had movement selectivity and similar maximum firing rates during the preferred movements. We recorded the activity of 107 M1 neurons before and after the injections (pre: n=62; post: n=45, recorded within 1-2 mm from the injection sites) and obtained the maximum mean firing rate and a selectivity index (SI) for each neuron (Fig. 5E and F) (see Methods). There were no significant differences in these measures between pre and post injections (*t-test*, $p<0.05$) (Fig. 5E and F). The results suggest that the deficit in task performance after the anisomycin injection was likely to be a specific consequence of its translational blockade, rather than any nonspecific inhibition or dysfunction of neural activity.

These observations show that we successfully manipulated the memory by injecting a protein synthesis inhibitor. The inhibition of protein synthesis in M1 disrupted the performance of memory guided sequential movements at all stages of learning without disrupting motor production. Observations from M1 inactivation using muscimol, recording of neural activity after the anisomycin injection and the injection of cycloheximide, another type of protein

synthesis inhibitor, all support the conclusion that the deficit in the performance of the Repeating task after the anisomycin injection was a specific consequence of the induced translational blockade (48, 53-55).

The effect of protein synthesis inhibition in M1 on the performance of the memory guided sequential movements decreased as learning progressed.

As previously demonstrated (Figs. 3 and 4), the effect of protein synthesis inhibition in M1 on the Repeating sequences varied depending on the training duration of each sequence. To test whether the effect of protein synthesis inhibition in M1 is correlated with the training duration, we repeated the anisomycin injection after the monkey's performance recovered to the pre-injection level and tested its effect on performance of the tasks (Fig. 6, Monkey 1, n=5; Monkey 2, n=4). The injection sites were all placed within the shoulder or elbow area in M1, but at slightly different sites to minimize the tissue damage. After each injection session, the monkey was trained daily, and its recovery of the task performance was confirmed (Fig. 6 A, B). The performance recovered after 2-3 days of training regardless of training duration. The injection sessions were separated by more than 2 weeks of training (see Methods). Overall, the effect of injections on the error rates of the Repeating task was strong and consistent. In contrast, the effect of injections on the performance of the Random task was consistently not significant.

We examined whether the effect of injection on performance depends on the training duration (Fig. 6C). The increase of error rate in the sequence with 'Short-term' training was significantly larger than that of the sequence with 'Long-term' training (Fig. 6C top, t-test, $p<0.05$). Similarly, the increase of the Response Time (RT) of the sequence with 'Short-term' training was significantly larger than that of the sequences with 'Long-term' and 'Extensive' training (Fig 6C bottom, t-test, $p<0.05$). However, the difference of increase of error rate and increase of RT between the sequences with the 'Long-term' training and 'Extensive' training was not statistically different (t-test, $p>0.05$).

We further examined whether the effect of injection on performance of the Repeating task is correlated with training duration. We found that the size of the injection effect on the RT of each movement is correlated with the training duration of a sequence (Fig. 6D; $p=0.001$). On the other hand, the size of the injection effect on the error rate of each movement was not correlated with the training duration of a sequence ($p>0.05$). The findings suggest that during the slow learning phase, the transition time from the movement to the next becomes short leading to the shorter RT.

The results show that the neural representation of 'Long-term' trained sequence and 'Extensively' trained sequence are less affected by the protein synthesis inhibition, and thus less vulnerable to interference. The decreased injection effects indicate that the rate of memory consolidation declines as learning proceeds. Our results suggest that the neural traces of sequential movements in M1 are gradually stabilized through repetitive practice to support a high level of performance.

Consolidation during repetitive practice: Response Time of the Repeating task before and after the anisomycin injection

The analysis of Response Time (RT) provided further insight into the consolidation process during repetitive training (Fig. 7; see Methods for RT definition). At the beginning of the post-injection session, RTs of Random and Repeating trials were similar to RTs of the pre-injection session (Random: ~450 msec; Repeating: ~ < 0 sec). Then, the monkey's RTs increased only in the Repeating tasks. For example, his RTs of the sequence '5-3-1' in the Repeating task (blue) increased incrementally in each block of Repeating trials, from below 0 msec to approximately 500 msec by the end of the session (Fig. 7 A, B) (t-test, $p<0.05$). On the other hand, RTs of the Random task did not change significantly within a session (Fig. 7B). This suggests that performing the task disrupted the neural representation of the Repeating sequences in M1. This is consistent with findings of rodent studies proposing that the synapses in the memory network would be first destabilized by protein degradation upon memory reactivation during task performance, and then stabilized to update the memory through protein synthesis (40, 41, 46, 47, 49, 50, 56-58).

We next asked whether exposure to anisomycin alone disrupts the performance of the Repeating task or whether performing the Repeating task within the drug effect window is necessary to disrupt performance of the Repeating task (Fig. 7C) (40, 41). We injected anisomycin into M1 of a highly trained monkey and assessed task performance after three days without training, at which time the injected anisomycin was no longer effective. Previous studies showed that protein synthesis level returned to normal 48 hours after an anisomycin injection (45). Task performance before the injection and after 3 days of inactivity (i.e., outside the drug effect window) was not significantly different for either the Random or Repeating tasks (Fig. 8C). The results suggest that exposure of M1 to anisomycin alone did not disrupt the task performance but performing the Repeating task within the drug exposure window was necessary to disrupt the neural representations of sequential movements stored in M1. Our data suggest that anisomycin prevented the synthesis of the proteins needed to consolidate the neural representations of sequential movements (i.e., synaptic connections) in M1 during task performance.

Discussion

In this study, we trained monkeys to learn sequential movements and interfered with information storage in the primary motor cortex (M1) at multiple time points during learning by injecting protein synthesis inhibitor, anisomycin, into the arm area of M1. The anisomycin injection disrupted the performance of memory guided sequential reaching at all stages of learning without affecting movement production. The effect of protein synthesis inhibition decreased as learning progressed.

Growing evidence showed that M1's activity changes with the learning of sequential

movements. The activity change within a subject (human or non-human primate) was reported in studies with training duration that ranged from within a training session to several months. Furthermore, the M1's changes after years of sequence training was reported in studies comparing musicians to non-musician or monkeys performing extensively trained sequential movements to those performing visually guided movements. However, it remains unclear in which learning stage plasticity occurs in M1. The change after extensive training could be the outcome of plasticity during the early phase of the training. Our study using continuous long-term training and the staggered introduction of sequences covered learning stages within individual monkeys. Our results demonstrated that the protein synthesis inhibition disrupted the performance of memory-guided sequential reaching tasks at all stages of learning examined. This suggests that plasticity in M1 occurs at all stages of learning during repetitive practice over an extended period, continuously supporting learning of the sequential movements.

The supplementary motor area (SMA) has long been considered responsible for the execution and maintenance of learned sequential movements (8, 16, 59-70). The SMA neurons exhibit sequence specific activity (60, 63, 71-78). Inactivation or lesion of the SMA disrupted the performance of memorized sequential movements (64, 79-84). In this view, M1 has been considered to relay the signal from SMA to execute the sequential movements. Additionally, inactivation of the dorsal premotor cortex (PMd) disrupted the performance of memorized sequential movements (85) and the PMd neurons exhibit sequence preferential activity (85). We cannot rule out that the SMA or PMd input to M1 (86-90) drives learning related changes in M1. However, our results suggest that aspects of learned sequences are consolidated within M1 at all times. M1's contributions could be to enhance synaptic efficacy for inputs from other areas to M1, improving fluency of the transitional movements between movement components within a sequence and improving the execution of individual movement components within a sequence. Further studies are needed to explore these possibilities.

Studies using protein synthesis inhibitors suggest that memory may be dynamically modified or reconsolidated upon retrieval (46, 47, 49, 91-93). Specifically, synapses within the memory network are first destabilized by protein degradation during reactivation, followed by stabilization to update or strengthen the memory through protein synthesis (46, 47, 49, 50). Supporting this view, the inhibition of protein degradation by clasto-lactacystin-β-lactone (βlac) has been shown to prevent anisomycin-induced memory impairment (94). Moreover, human studies using TMS and imaging suggest that memory reconsolidation may occur in humans as well (95-99). Several factors, including the age and strength of a memory (56, 91, 100), the novelty of the experience (101-103), and duration of memory reactivation necessary for reconsolidation to occur (56, 57) are proposed as boundary conditions, which are considered to influence whether memory reconsolidation occurs.

In our study, we observed that the performance of the Repeating task was disrupted with a delayed onset of deficits after the anisomycin injection. The performance deficits may result from interference with the reconsolidation occurring during repetitive practice. The delayed onset of deficits may be linked to the duration of memory reactivation necessary for reconsolidation to occur (56, 57), specifically the duration for protein degradation to destabilize

the memory network for reconsolidation.

Previous studies have shown that memory could be updated through reconsolidation when there was a new information to integrate (104-106) and that the age and strength of memory have an impact on whether reconsolidation occurs or not. In other words, old and strong (i.e., trained more than certain period of time) memories were less sensitive to protein synthesis inhibition. In our study, the effect of protein synthesis inhibition on memory-guided sequence performance was smaller during the 'Long-term and 'Extended' training phases in which RT variability was smaller. In our task, performance changes due to movement variability could constitute the equivalent of novelty as a boundary condition for reconsolidation. Our results suggest that the memory network in M1 may be destabilized prior to reconsolidation (or update) at every practice rather than building incrementally. This is consistent with a 'lingering consolidation' hypothesis, in which 'the reactivation and reconsolidation cycle progressively stabilizes a memory' (47).

Two-photon imaging studies offer insights into the possible mechanisms underlying reconsolidation in the motor cortex. In rodents, two photon imaging of the motor cortex during motor skill learning has shown the formation and growth of new dendritic spines and the elimination of old spines (107, 108). Additionally, two-photon imaging of cortical slices and other studies revealed that protein synthesis is required for long-lasting synaptic plasticity (48) and spine-head enlargement and growth during learning (109-112). The inhibition of protein synthesis resulted in a significant reduction in synapse number and synapse size in M1 in vivo (44). These observations suggest that, during motor skill training, unnecessary spines are eliminated through protein degradation, while the formation and growth of new spines are supported by protein synthesis which leads to enhanced neural efficacy. Thus, deficits in spine growth and formation may be associated with impairments in memory reconsolidation, as observed in behavioral tests. Together with our findings, these results suggest that the neural trace for motor skills is continuously updated during repetitive practice through protein synthesis. The delayed onset of performance deficit, as shown in Figure 7, may reflect the time required for protein degradation to eliminate a sufficient number of spines to impact task performance.

Our study suggests that the motor skill is gradually consolidated in M1 every time a subject practice and the rate of consolidation (i.e., memory strengthening) becomes smaller as the learning proceeds. The lingering consolidation process implies that reconsolidation reaches a theoretical limit when the memory trace is old and has become stable. Nevertheless, our results suggest that reconsolidation still occurs in M1 in our monkeys after 377 sessions of practice on simple sequences. M1 may well make a steady contribution to the development of expertise.

Materials and Methods

Behavioral task: We trained two monkeys (*Cebus apella*, male or female, weighing 1~5 kg; > 2 years old) on the Random and Repeating tasks (Fig. 1) (37, 38, 52, 85, 113, 114). Each monkey was required to make sequential reaching movements to targets on a touch sensitive monitor with their right arm. In the Random task, the reaching movements were guided by visual

targets displayed on a touch sensitive monitor in a pseudo-random order. Contact of the correct target triggered display of the next target after a 400 ms delay. After the monkey became proficient in performing the Random task (~50 days of practice), we introduced the Repeating task. In the Repeating task, new targets were presented according to a repeating sequence of 3 elements (e.g., 5-3-1-5-3-1- ..., Fig. 1A, B) 400 msec after contact of the preceding target. If the monkey made a correct response during the delay, the target is not shown and the task increments to the next target in the sequence. With practice, a monkey performed the correct sequence in the Repeating task using predictive responses instead of visual cues to direct their movements in > 80% of trials. The two tasks were performed continuously in alternating blocks of 200-500 trials. The monkey received a liquid reward after every 4-5 correct responses. After the monkey became proficient in the performance of the 1st sequence (Fig. 1C), additional sequences (e.g., 1-2-4-1-2-4...) were introduced. Introduction of the new sequences was staggered at ~ 50-200 days intervals (Fig. 1D). This training design enabled us to compare the effect of pharmacological injection between the 'Long-term' trained and 'Short-term' trained sequences in a single injection session. Control of the behavioral task and collection of movement data including videos of task performance were performed by PC computers running TEMPO software (Reflective Computing, Olympia, WA) and Matlab. Touch screen signals, task related events and EMG activity were collected on-line at 1 kHz.

Behavioral estimates of learning phases: We used "Response Time" (RT) as the principal measure of sequence learning. RT was defined as the time between two target touches minus the delay time. During the learning, an animal's RT became shorter with practice (Fig. 7c, d). A negative RT indicates that the animal was internally generating the response before the visual cue was presented. After ~50 days of training on the Repeating sequence, the monkey made predictive responses (RT < 150 ms) without using visual cues in more than 80% of the trials (Fig. 1C, D).

Surgical Procedures: All experimental procedures were conducted according to NIH guidelines and were approved by the IACUC of the University of Pittsburgh. We implanted a head restraint device, along with a MR compatible chamber for micro-injections, on an animal's skull using small screws and dental acrylic (85). All surgical procedures were performed under general anesthesia using aseptic techniques. Anesthesia was induced with ketamine (10 mg/kg, IM) and maintained to surgical levels with 1-2.5% isoflurane. The animal received fluids throughout the surgical procedure (6-10 cc/hr, IV) and glycopyrolate (0.01 mg/kg, IM) to reduce secretions. Body temperature was maintained at 37-38 °C with a heating pad. We monitored body temperature, breathing, heart rate, end-tidal CO_2, and O_2 saturation. After the surgery, the animal received broad spectrum antibiotics (ceftriaxone, 75 mg/kg/day, IM, ~5 days) and analgesics (buprenorphine, 0.01 mg/kg, IM, ~3 days). The chamber's placement over M1 was verified using structural MR images taken prior to and after the surgery (fig. 2). MR procedures for pretreatment, anesthesia, monitoring of vital signs and post-anesthesia monitoring were identical to surgical procedures, except that analgesic and antibiotic were not given. After more than 2 weeks of recovery, we accustomed the animal to perform the tasks with its head restrained. When task performance returned to the pre-surgical level, we performed a craniotomy to expose

the cortex in the chamber using the same anesthetic techniques and safeguards used in the implant surgery. Animals were given dexamethasone (0.5 mg/kg divided into 3 doses, IM) on the day of the craniotomy surgery to prevent brain swelling.

Intracortical stimulation: We used intracortical microstimulation to identify the body part represented at each site in M1 and to physiologically define the border between M1 and the PMd (52, 85, 90). We used glass-coated micro-electrodes (0.6-2 MΩ at 1 kHz) to deliver intracortical microstimuli (115). A constant-current stimulator was used to deliver cathodal pulses (10–20 pulses, 0.2 ms duration, 333 Hz, 1- 40 µA) at a depth of 1500 µm below the cortical surface. 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 more than 50% of the trials (90, 116, 117). We systematically mapped M1 with micro-electrode penetrations spaced ~1.0 mm apart (except to avoid blood vessels) (Fig. 2).

Micro-injections: We made micro-injections of pharmacological agents (anisomycin, cycloheximide or muscimol) at selected sites in the forelimb area of M1 of a trained monkey (52, 85). We prepared solutions of anisomycin (100 µg/µl in ACSF, pH 7.2-7.4), cycloheximide (40 µg/µl in 20% ethanol/ACSF) and muscimol (5 mg/ml in saline) from commercially available powders (Sigma-Aldrich, MO). We injected 1-5 µl of the test solution at 1.5 mm below the cortical surface using a 30-gauge cannula connected to a 10 µl Hamilton syringe by applying slow pressure (e.g., over 10 minutes). More than 3 µl of pharmacological agent were divided and injected into 2 sites. An injection site was placed more than 2 mm away from the border between M1 and PMd identified by microstimulation (Fig. 2). Previous studies using monkeys reported that infusion of 3 µl of muscimol into cortex inhibited the activity of neurons within a diameter of 2-3 mm (84). Because M1 in Cebus is more than 5 mm in width (Fig. 2), the effect of 3 µl of a pharmacological agent was limited to M1 (52). The cannula was left in place for more than 2 min to allow diffusion of the solution and prevent its reflux. We determined whether the injection of the pharmacological agent impairs performance of the two tasks. The effect of anisomycin injection on task performance was tested 20-24 hours after the injection and every day thereafter. The injections were done multiple times during learning. The effect of cycloheximide injection on tasks was tested 20-24 hours after the injection. Cycloheximide blocks protein synthesis by a different mechanism than anisomycin. We verified that the effect on behavior is due to the common effect of the agents (i.e., protein synthesis inhibition). Muscimol, $GABA_A$ agonist, (1-3 µl) was injected immediately after the collection of the baseline behavioral data and the effect of injection on task performance was tested 20 min after the injection to allow diffusion of the chemical in the brain tissue.

After each injection session, the task performance was monitored every day until it recovered to the baseline to track the emergence of injection effects and to assess recovery from the drug's effects. Injections were separated with more than 7 days and 5 training sessions. During a post-injection test session, blocks of Random and Repeating trials 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 session, we recorded various task parameters and measures of performance. The effect of an injection was assessed by examining the following: percentage of correct responses, types of incorrect responses, movement trajectory, percentage of predictive responses (defined as RT < 150 msec), and Response Time (RT) and Movement Time (MT) (52, 85). The errors recorded were: no hit, wrong target hit, background hit, or a corrective response (a correct response that immediately follows an error). A wrong target hit during the Repeating task was categorized as two types: *accuracy errors* and *direction errors*. An *accuracy error* is defined as a reach performed in the correct direction, but to an endpoint outside of the correct target. This type of error suggests a deficit in motor production. A *direction error* was defined as a reach performed in the direction opposite to the correct target. This type of error suggests a deficit in selecting the movement component in the sequence. An increase in RT and decrease in the number of predictive responses suggest an increase in the time for movement selection. Corrective responses were removed from analysis because the target is predictable after an error. We also monitored movement kinematics during the task using high speed video recording (100 Hz) in the frontal and sagittal planes (Basler Inc., PA; Advanced Digital Vision Inc., MA). For statistical analysis, we used χ^2 tests with Holm–Bonferroni's correction to examine the significance of changes in success rate and predictive responses in a session. We used *t*-tests with Holm–Bonferroni's correction to examine MT and RT changes. We used *t*-tests with Holm–Bonferroni's correction to examine population data obtained in multiple injection sessions. For each animal, we repeated the anisomycin injections more than 3 times to test the reproducibility of results within an animal. We compared the strength of the injection effect between the sequences in the different learning phases (e.g., 'Long-term' vs 'Short-term').

Electrophysiological recording and analysis of neural activity: To evaluate the effect of pharmacological agents on neural activity, we recorded extracellular activity of single neurons in M1 using glass-coated Elgiloy electrodes (0.6-1.5 MΩ). We recorded neural activity before and after the injection of anisomycin while a monkey performed the tasks. As shown in fig. ###, an anisomycin injection disrupted performance of the Repeating task on up to 80% of trials. Therefore, to evaluate the effect of injection on neural activity, we limited analysis on the data collected during the Random task. The sample of neurons was taken within 2 mm of the injection site from regions of M1 where intracortical stimulation evoked shoulder, elbow or wrist movements at thresholds lower than 30 µA. For each neuron, we used maximum firing rate and a selectivity index (SI) to assess the effect of injections. For each Random move, we measured the mean firing rate of a neuron in a 200 msec interval centered on target contact or release. We used this value to calculate a SI for each neuron: SI = [Max - Min]/ [Max + Min]. For statistical analysis, we used *t-test* to examine changes in SI and max firing rate between the group of M1 neurons recorded before and after the injections.

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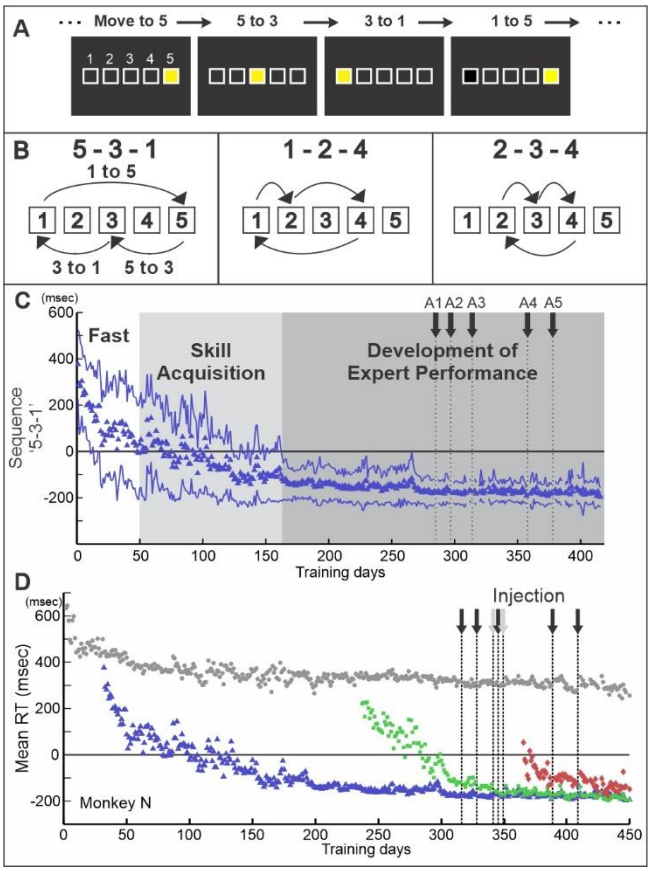

Fig. 1 Sequential Reaching Task. A. Sequential reaching task. B. Sequences in the Repeating task. C. Response Time (RT) of sequence '5-3-1' of the Repeating task. We arbitrary divided the learning curve on the Repeating task into 3 periods based on the slope of RT changes. The first period corresponds to the 'Fast Learning' phase where a large RT reduction occurs in a short time frame. The second and third periods are defined as the early and late epochs of the Slow Learning phase and correspond to 'Skill Acquisition' and the 'Development of Expert Performance'. D. The introductions of the new sequences were staggered at ~ 100-200 days intervals. This staggered training design enabled us to compare the effect of pharmacological injections between the 'Long-term' trained and 'Short-term' trained sequences in a single injection session. Gray circle: RT of the Random task; Blue triangle: RT of the Repeating sequence "5-3-1"; blue lines in C: mean +/- s.d.; Green square: RT of the Repeating sequence "1-2-4"; Red diamond: RT of the Repeating sequence "2-3-4': Arrow: injection of anisomycin.

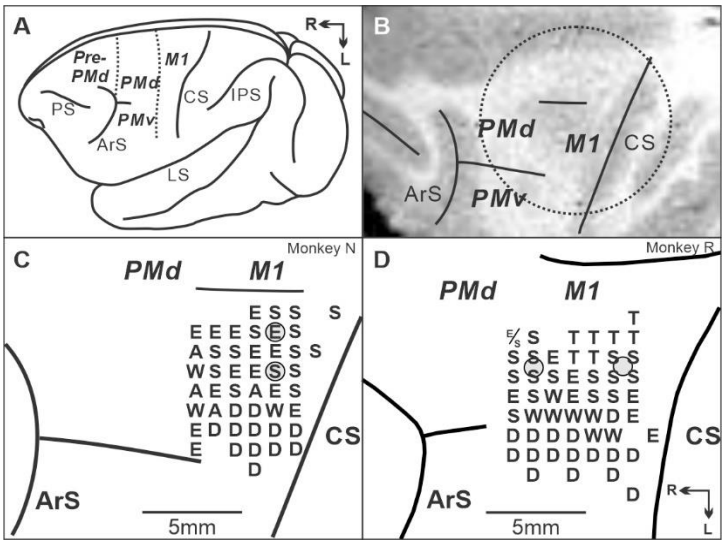

Fig. 2 M1 location and the microstimulation maps of M1. A. Lateral view of cebus brain. Dashed lines indicate the M1-PMd border and the pre-PMd-PMd border. PS: principal sulcus; ArS: arcuate sulcus; CS: central sulcus; IPS: intra parietal sulcus; LS: lateral sulcus; pre-PMd: pre-dorsal premotor cortex; PMd: dorsal premotor cortex; M1: primary motor cortex. R: rostral; L: lateral. B. MRI image after the chamber implantation for monkey N. The dotted circle indicates the chamber outline. C, D. Intracortical stimulation map from monkey N and monkey R. Letters indicate the movements evoked at each site. S: Shoulder; E: Elbow; W: Wrist; D: Digit; Gray dots: representative injection sites.

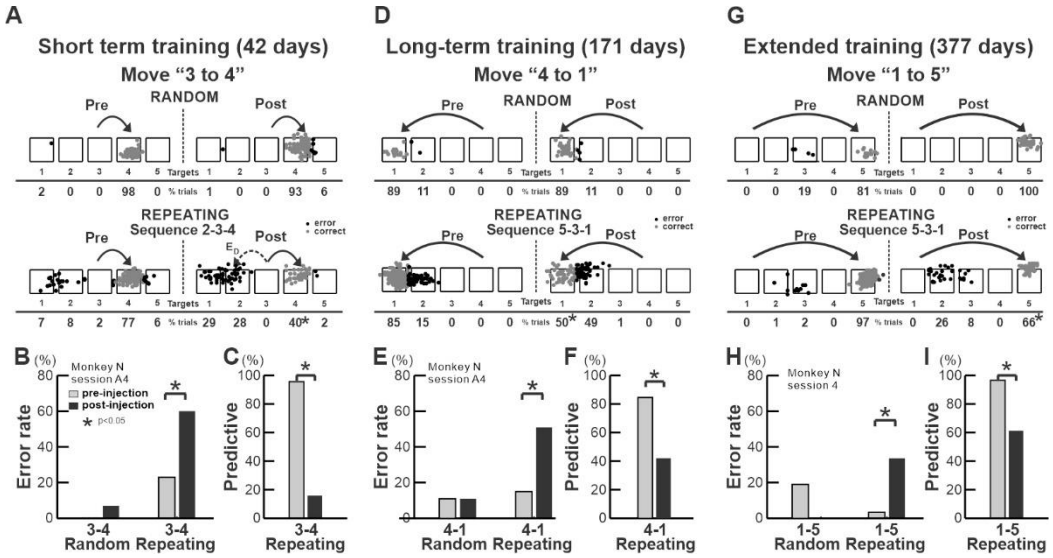

Fig. 3 Effect of anisomycin injection on performance of the Random and Repeating tasks. A-C. Effect of ANI injection on movements from target "3 to 4" in the sequence "2-3-4" of the Repeating task after 42 days of training. A. Reaching end points for movements from target 3 to 4 ('3-4") during the Random and Repeating tasks. Left: pre-injection; Right: post-injection. E_D: direction errors. Gray dots: correct responses; black dots: error responses. Percentages of reaches ending at each target are shown below the targets. Touches between targets were counted as touches to the closest target. B. Error rate of movement "3-4" in Random and Repeating tasks. Errors increased only in the Repeating task after ANI injection (χ^2 test, $p < 0.05$). C. Predictive responses decreased significantly after injection (χ^2 test, $p < 0.05$). D-F. Effect of ANI injection on movements from target "4 to 1" in the sequence "1-2-4" of the Repeating task after 171 days of training and movements "4-1" of the Random task. D. Left: pre-injection; Right: post-injection. E_A: accuracy errors. Gray dots: correct responses; black dots: error responses. E. Error rate of movements "4-1" in Random and Repeating tasks. Errors increased only in the Repeating task after injection (χ^2 test, $p < 0.05$). F. Predictive responses of movements "4-1". G-I. Effect of ANI injection on movements from target "1 to 5" in the sequence "5-3-1" of the Repeating task after 377 days of training and movements "1-5" of the Random task. G. Left: pre-injection; Right: post-injection. EA: accuracy errors. Gray dots: correct responses; black dots: error responses. H. Error rate of movements "1-5" in Random and Repeating tasks. Errors increased only in the Repeating task after injection (χ^2 test, $p < 0.05$). I. Predictive responses of movements "1-5". Note: * indicates $p < 0.05$.

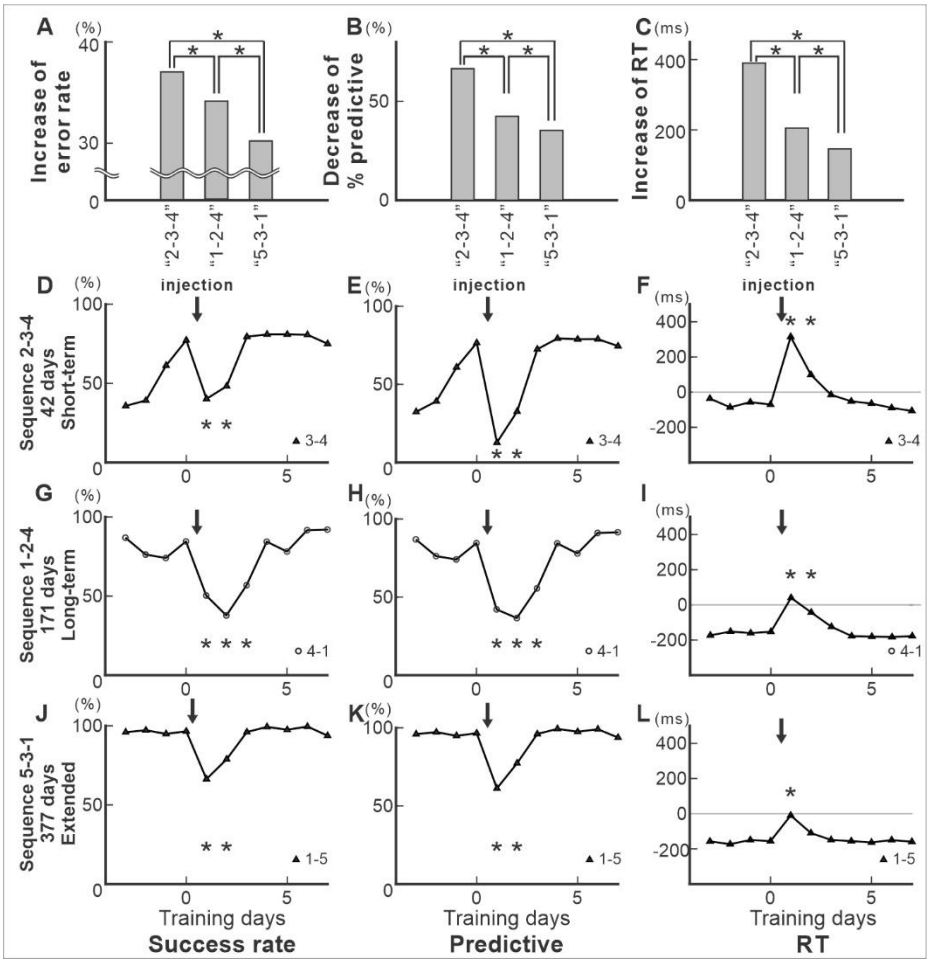

Fig. 4 Performance of the Repeating task for the injection session shown in Fig. 3. A. Increase in error rate across three sequences in the Repeating task. B. Decrease in the percentage of predictive trials. C. Increase in Response Time (RT). D-L. Performance of the Repeating task from 4 days before to 7 days after anisomycin injection. The anisomycin injection was made at the time points indicated by arrows. Anisomycin injection in M1 impaired performance in the Repeating task for 2-3 days post-injection, even after 'Extensive' training. Task performance returned to baseline after 2-3 days of retraining. Asterisks indicate significant differences from baseline (χ^2 test, $p < 0.05$).

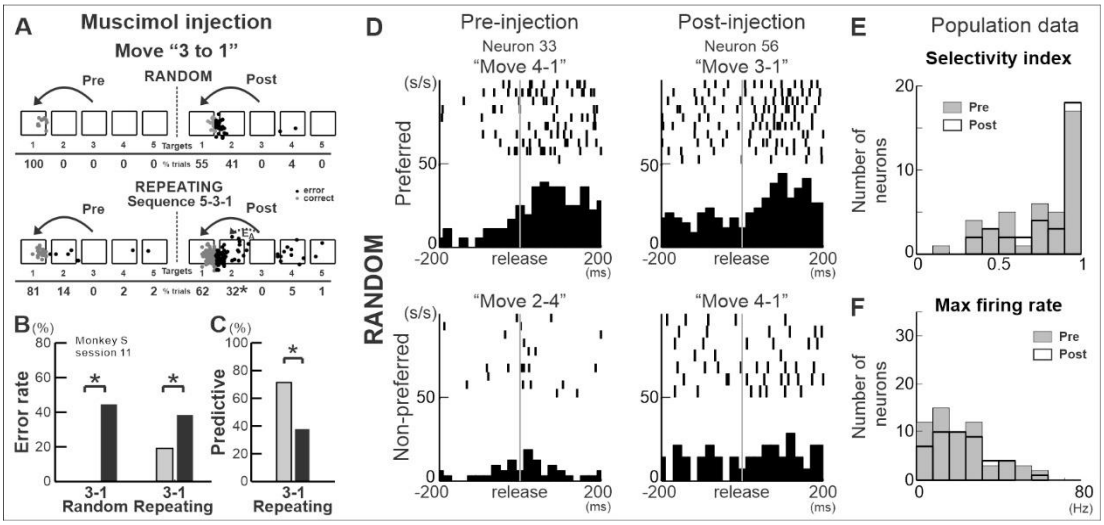

Fig. 5 Verified that the observed behavioral effects (Fig. 3) were specifically due to protein synthesis inhibition affecting memory,A-C, The effect of muscimol injection in M1 on movements from target "3 to 1" of the Random and Repeating tasks. A. Left: pre-injection; Right: post-injection. gray: correct: black: error. B. Error rates. The number of errors increased both in the Random and Repeating tasks after the injection ($\chi 2$ test, $p<0.05$). C. Predictive responses. * = $p<0.05$. D. Activity of 2 individual M1 neurons. Neural activity was aligned at the monkey's release of the touch screen. Left: Activity of a neuron recorded before the anisomycin injection (neuron 33). Right: Activity of a neuron recorded 24 hours after the anisomycin injection (neuron 56). Upper panels: neural activity during the movements that had maximum firing rates (Neuron 33: during movement from target 4 to 1; Neuron 56: during movement from target 3 to 1). Lower panels: neural activity during the movements that had minimum firing rates (Neuron 33: during movement from target 2 to 4; Neuron 56: during movement from target 4 to 1). Both neurons were recorded 1 mm away from the injection site. Both neurons had movement selectivity in the Random task. E. Selectivity index of M1 neurons recorded before and after the anisomycin injections (pre n=62; post n=45). There was no significant difference of selectivity indexes between pre- and post- injections. F. Max firing rate of M1 neurons recorded before and after the anisomycin injections (pre: n=62; post: n=45).

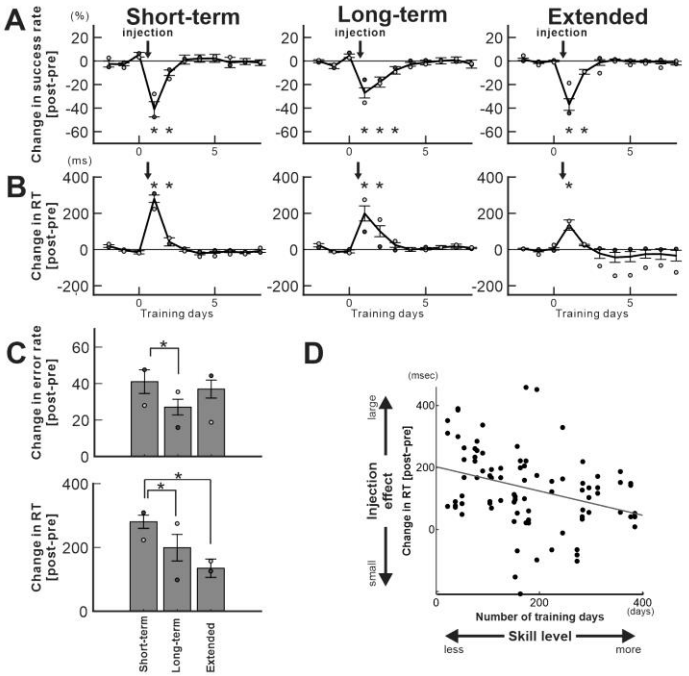

Fig. 6 The effect of anisomycin injection on performance of the Repeating task (population data). A. Mean error rates of the most affected movements in the Repeating task from 3 days before to 8 days after anisomycin injection (monkey 1, n=5; monkey 2, n=4). Arrows indicate a time point of the anisomycin injection. Light gray dot: mean error rate of monkey 1; dark gray dot: mean error rate of monkey 2; error bars: se. B. Mean Response Time (RT) of most affected movement in the Repeating task from 3 days before to 8 days after anisomycin injection (monkey 1, n=5; monkey 2, n=4). Arrows indicate a time point of the anisomycin injection. Light gray dot: mean error rate of monkey 1; dark gray dot: mean error rate of monkey 2; bars: se. Anisomycin injection in M1 affected error rate and RT in the Repeating task for 2-3 days post-injection, even after 'Extensive' training. Task performance returned to baseline after 2-3 days of retraining. Asterisks indicate significant differences from baseline (χ^2 test, $p < 0.05$). C. Mean change of error rate (top) and increase of RT for sequences with different training duration after the anisomycin injection. Light gray dot: mean of monkey 1; dark gray dot: mean of monkey 2; error bars: s.e. D. The size of the effect of injection on RT is correlated with the number of training days (r=-03277, p=0.0019). The x-axis represents the effect of the injection on RT, while the y-axis indicates the number of training days.

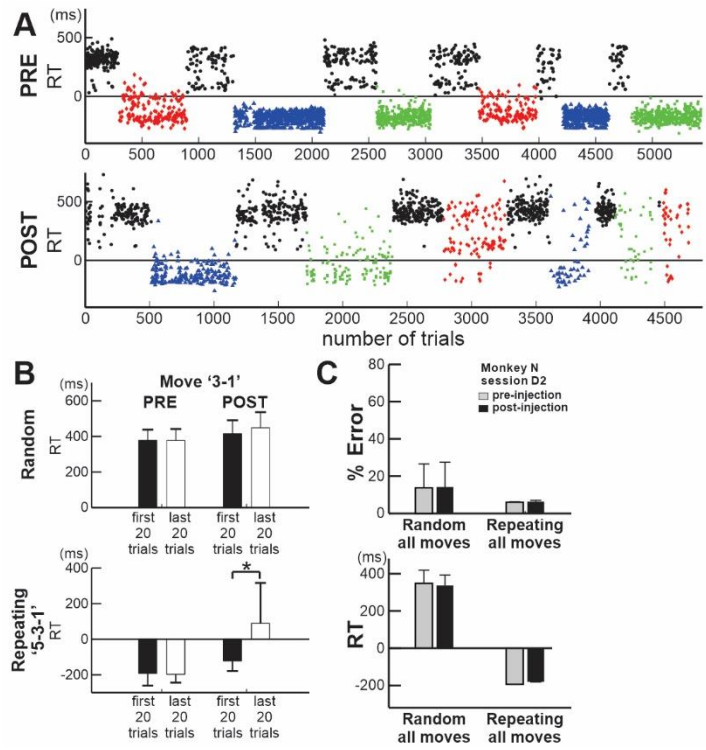

Fig. 7 The effect of anisomycin injection on Response Time (RT). A. RT for each trial of the Random (black) and the Repeating tasks (sequence '5-3-1': blue triangle; '1-2-4': green square; '2-3-4': red circle) before (top) and after (bottom) an anisomycin injection. The RT of 3 sequences increased during the post- anisomycin injection session. B. Mean RT of first and last 20 trials of movement '3-1' of the Repeating sequence '5-3-1' during pre and post injection sessions. * $p<0.05$, t-test. C. Performance test 3 days after the anisomycin injection. Top: Mean Error rate before and 3 days after the injection. There was no significant effect of injection on performance of the tasks ($\chi 2$ test, $p>0.05$). Bottom: Mean Response Time (RT) before and 3 days after the injection. There was no significant effect of injection on RT (t-test, $p>0.05$). All error bars are s.e.