

# Role of Monkey Cerebellar Nuclei in Skill for Sequential Movement

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**Lu, Xiaofeng, Okihide Hikosaka, and Shigehiro Miyachi.** Role of monkey cerebellar nuclei in skill for sequential movement. *J. Neurophysiol.* 79: 2245–2254, 1998. To examine whether the cerebellum is involved in learning and memory of visuomotor sequences, we trained two monkeys on a sequential button press task and inactivated different portions of the cerebellar nuclei by injecting a small amount of muscimol ( $\gamma$ -aminobutyric acid agonist). Before the injection experiments started, the monkeys had learned a set of sequences ( $n = 21$  and  $12$ ) extensively. After each injection, we had the monkeys perform the learned sequences and, in addition, learn new sequences. We found deficits in learning/memory by the injections into the dorsal and central part of the dentate nucleus. The number of errors increased significantly for the learned sequences but not for the new sequences. This effect was present only when the hand ipsilateral to the muscimol injection was used. Consistent with this result, anticipatory saccades, the occurrence of which is correlated closely with motor skill, also became less frequent particularly when the ipsilateral hand was used. No effect on learning/memory was observed after injections into the ventral or lateral parts of the dentate nucleus, interpositus nucleus, or fastigial nucleus. In contrast, hand movements became slower after ipsilateral injections at all of the injection sites. These results suggest that, among the cerebellar nuclei, the dentate nucleus, especially its dorsal and central regions, is related to the storage and/or retrieval of long-term memory for motor skill.

## INTRODUCTION

When we learn a complex action by repeating it, the performance becomes increasingly automatic and stereotyped (Fitts 1964); this often is called a "motor skill." This observation implies that a neural circuit is created and/or strengthened during practice which reliably produces movements constituting the skill. Searching for the newly created circuit (called memory or engram) underlying such motor skills has been a major issue in neuroscience. A long-standing hypothesis is that the cerebellum is the site for motor skill memory (Ito 1984; Thach et al. 1992). Cerebellar dysfunction, either clinically or experimentally induced, is characterized by a lack of coordination (asynergia) across muscles (Soechting et al. 1976), joints (Thach et al. 1992), effectors (e.g., eye-hand) (Vercher and Gauthier 1988), and time (i.e., sequencing) (Keele and Ivry 1990). Cerebellar dysfunction also can prevent adaptive modifications, such as vestibuloocular reflex (Robinson 1976), saccadic eye movements (Optican and Robinson 1980), and prism adaptation (Baizer and Glickstein 1974). Support for the cerebellar theory comes from studies on eyelid response conditioning (Krupa et al. 1993; McCormick and Thompson 1984) and the discovery of long-term depression (LTD) in Purkinje cells (Ito 1989), in addition to theoretical studies (Kawato and Gomi 1992; Raymond et al. 1996). Furthermore, recent human imaging studies have shown that the cerebellum is

activated during motor or procedural learning (Flament et al. 1996; Jenkins et al. 1994; Jueptner et al. 1997; Kim et al. 1994; Seitz et al. 1994) and that cerebellar patients are impaired in performing procedural learning tasks (Molinari et al. 1997; Pascual-Leone et al. 1993; Sanes et al. 1990). However, there have been few animal studies that examined the learning and memory mechanisms for such complex movements (Nixon and Passingham 1996).

A sequential button-press task developed in our laboratory for monkey and human subjects (Hikosaka et al. 1995) is suitable for the experimental approach to this issue. The task, which we call "2  $\times$  5 task," requires the subject to find the correct order of button presses for five consecutive pairs of targets. With long-term practice for a particular sequence, monkey subjects become able to perform the task very skillfully and eventually acquires visuomotor skills for many different sequences. Once having acquired the skill, monkeys can retain the ability for a long time without practice (Hikosaka et al. 1996a).

In our laboratory, it was found that the medial premotor cortex, especially the presupplementary motor area (Miyashita et al. 1996b; Miyashita and Hikosaka 1997), together with the anterior striatum, is crucial for learning new sequences, whereas the posterior striatum is involved in memory storage or retrieval (Miyachi et al. 1997). The present study investigated the role of the cerebellum in learning and long-term memory of visuomotor sequences.

## METHODS

### *Experimental animals*

We used two male Japanese monkeys (*Macaca fuscata*): monkey KZ (8.6 kg) and monkey MC (9.5 kg). The monkeys were kept in individual primate cages in an air-conditioned room where food was always available. At the beginning of each experimental session, they were transported to the experimental room in a primate chair. The monkeys were given restricted amount of fluid during periods of training and recording. Their health conditions such as body weight and appetite were checked daily. Supplementary water and fruit were provided daily. All surgical and experimental protocols were approved by the Juntendo University Animal Care and Use Committee and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

### *Apparatus*

The monkeys were trained to perform a sequential button press task called 2  $\times$  5 task (see Hikosaka et al. 1995, for details). Briefly, the monkey sat in a primate chair and faced a panel on which 16 LED buttons were mounted in a 4  $\times$  4 matrix. At the bottom of the panel was another LED button that was used as a home key. To have the monkey use only one hand for button press,

a vertical Plexiglas plate was attached to the chair in an oblique direction between the panel and the hand not being used. To change hand for performance, the plate was rotated to the other side.

**Behavioral paradigm**

**2 × 5 TASK.** The 2 × 5 task was to press five consecutive pairs of buttons in correct orders, which the monkeys had to find by trial-and-error in a block of trials. Figure 1 shows an example of the sequence of events in a single task trial. At the start of a trial, the home key illuminated. When the monkey pressed the home key for 1 s, two of the 16 target LEDs turned on simultaneously, which we call “set.” The monkey had to press the illuminated buttons in the correct (predetermined) order, which he had to discover by trial and error. If successful, each button would turn off when depressed. If both were correct, another pair of LEDs, a second set, turned on, and the monkey had to press again in the correct order. A total of five sets were presented in a fixed order for completion of a trial, which we call “hyperset.” When the animal pressed a wrong button, the trial was aborted without any reward. The animal then had to start again from the home key as a new trial. It should be emphasized that a trial was determined to be successful only when the monkey completed the whole hyperset (5 sets). The monkey was given a liquid reward after each success-

ful set. The amount of reward was increased toward the final set to encourage the monkey to complete the whole hyperset. The same hyperset was repeated throughout a block of experiment until the monkey completed 10 trials successfully. A different hyperset then was used for the next block. Successive trials were separated by an interval of 0.5–3 s by inactivating the panel.

**NEW AND LEARNED SEQUENCES.** We hypothesized that there are separate neural mechanisms underlying procedural learning and memory, one for acquisition of new procedures and the other for storage of long-term memories and their retrieval. Prerequisite for testing this hypothesis was the experimental situation in which the monkey had acquired long-term procedural memories and at the same time had opportunities to learn new procedures repeatedly.

To create long-term memories, we asked the monkeys to practice a set of hypersets (21 for *monkey KZ* and 12 for *monkey MC*) almost every day for >12 mo for *monkey KZ* and 11 mo for *monkey MC* before recordings started. As a result, the monkeys became very skillful in performing them and they were termed “learned hypersets.” About one-half of the learned hypersets were performed always by the right hand, the other half by the left hand.

In addition, we had the monkeys learn newly computer-generated hypersets (new hypersets). *Monkeys KZ* and *MC* had experienced >1,400 and 1,200 new hypersets respectively before re-

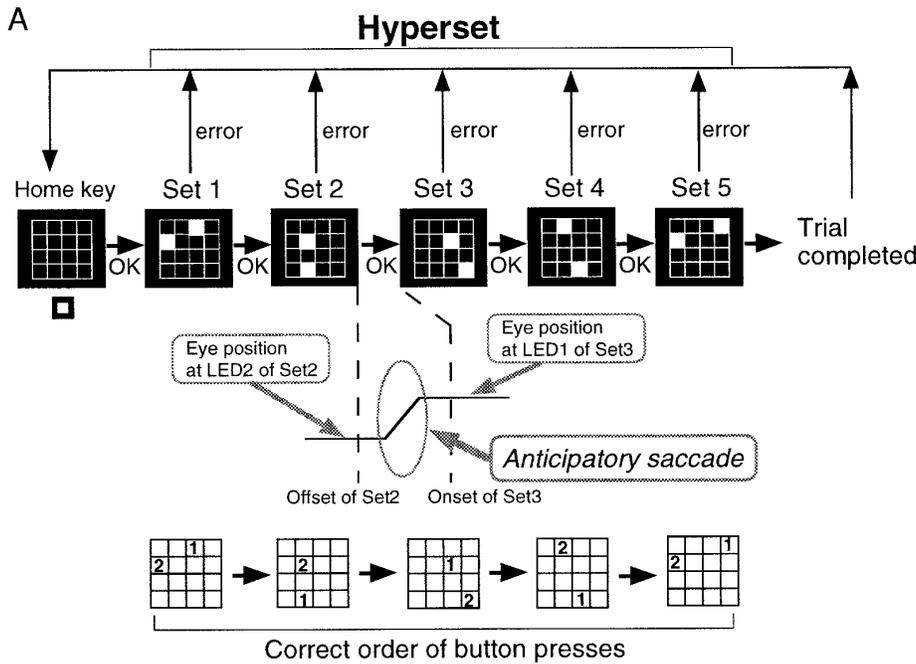
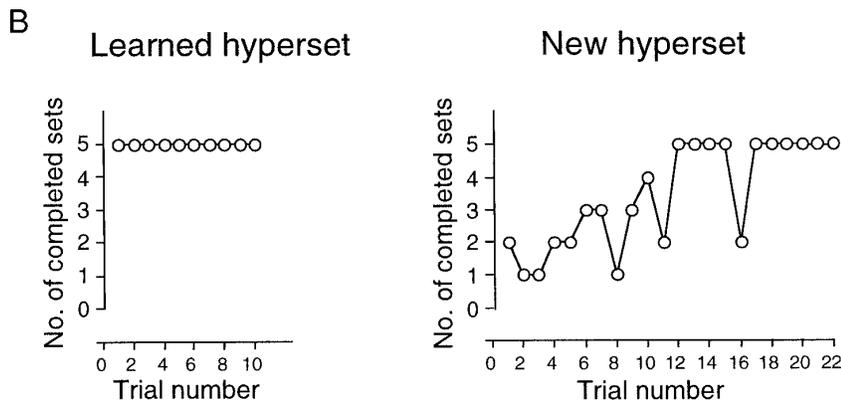


FIG. 1. A: example of hyperset in the 2 × 5 task. To complete a trial, the monkey had to press 10 buttons (2 buttons × 5 sets) in the correct (predetermined) order. On pressing of the home key, 2 of 16 LED buttons were illuminated simultaneously (set 1), and the monkey had to press them in the correct order. If successful, pairs of LED buttons were illuminated sequentially (sets 2–5), and the monkey had to press the 2 buttons in each pair in the correct order. Whole sequence was called a hyperset. Trial was regarded successful only when the whole hyperset was completed. If the monkey pressed a button in a wrong order in any set, the trial was aborted (error trial), and he had to restart from set 1. B: performance of *monkey KZ* for a learned hyperset (left) and a new hyperset (right). Number of completed sets (ordinate) is shown for the consecutive trial number (abscissa). Within a block of experiments, the same hyperset was repeated until the monkey completed it successfully 10 times. In these examples, the number of errors in a block of experiments was 0 for the learned hyperset and 12 for the new hyperset. This parameter was used to quantify the grade of learning. Before the injection experiments started, we had the monkeys perform a set of learned hypersets ( $n = 21$  for *monkey KZ*,  $n = 12$  for *monkey MC*) almost every day for 11–12 mo. New hypersets, which the monkeys had never experienced before, were used only once (for 1 block).



cordings started. Most of them were performed just once (1 block), and half of them were performed by the right hand, the other half were by the left hand. Thus the monkey experienced roughly an equal amount of practice for each hand throughout the learning period.

**SIMPLE REACTION TASK.** In addition to  $2 \times 5$  task, we used a visually guided, simple reaching task ("simple reaction task"). After pressing the home key for 1,000 ms, 1 of 16 buttons turned on. The monkey was required to press the illuminated button to obtain reward. The location of the target was chosen pseudorandomly such that each 1 of 16 targets appeared once for 16 consecutive trials.

### Surgery

The experiments were carried out while the monkey's head was fixed and eye movements were recorded. For this purpose, a head holder, chambers for unit recordings and drug injections, and an eye coil were implanted. The monkey was sedated with ketamine (4.0–5.0 mg/kg) and xylazine (1.0–2.0 mg/kg) intramuscularly, and then general anesthesia was induced by intravenous injection of pentobarbital sodium ( $5 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ ). Surgical procedures were conducted in aseptic conditions. After exposing the skull, 15–20 holes were drilled and tapped approximately three-fourths of the way through it. Acrylic screws ( $3 \times 6 \text{ mm}$ ) were inserted and fixed with dental acrylic resin. The screws acted as anchors for the dental acrylic, which held a head holder and two chambers, all made of delrin, to the skull. A scleral eye coil was implanted in one eye for monitoring eye position (Judge et al. 1980; Robinson 1963). Each of the recording/injection chambers was tilted laterally by  $35^\circ$  from vertical, while their centers were aimed at the dentate nucleus according to the atlas of Kusama and Mabuchi (1970). The monkey received antibiotics (sodium ampicillin 25–40 mg/kg im each day) after the operation.

### Implantation of guide tubes

The cerebellar nucleus first was determined electrophysiologically by recording single cell spike activities related to button press movements. After the cerebellar nuclei were mapped thoroughly by unit recordings, we implanted teflon-made guide tubes (Teflon, 0.8 mm ID, 1.1 mm OD), which were aimed at a particular portion of the cerebellar nuclei. They were fixed on the skull using dental acrylic resin so that their tips were positioned  $\sim 2 \text{ mm}$  beneath the surface of the cerebellar cortex. To facilitate the comparison between two monkeys, we intended to make the placement of the guide tube in the second monkey identical with that in the first monkey. For this purpose, we first made unit recordings at  $>50$  sites for each monkey so as to estimate the whole contour of the dentate nucleus. Then we implanted a guide tube at the intended location. In addition, we confirmed the correct placement of the guide tube using magnetic resonance imaging (MRI; Hitachi Laboratory MRIs, 2.11 tesla; Hitachi AIRIS, 0.3 tesla) before the injection experiments started. The positions of the guide tubes also were confirmed by histological examinations (see Fig. 2).

### Injection procedures

**DRUG.** We used a GABA agonist, muscimol (Sigma), to inactivate local brain activity. Muscimol was dissolved in saline with a concentration of  $5 \mu\text{g}/\mu\text{l}$  and was injected locally in different portions of the cerebellar nuclei, especially the dentate nucleus (see Fig. 2). All injections were carried out with an injection tube (see further) that was inserted into the cerebellum through the guide tube using a recording manipulator.

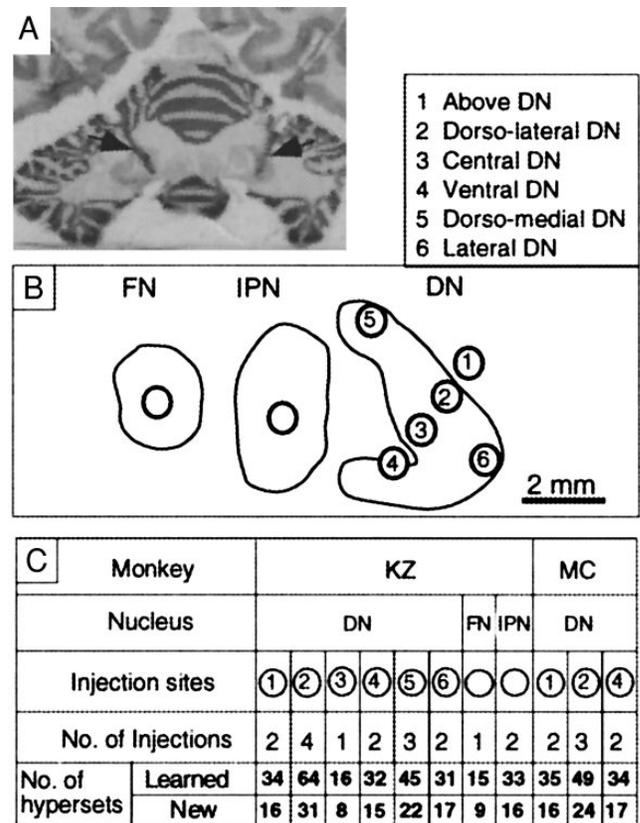


FIG. 2. Sites of muscimol injections in the cerebellar nuclei. *A*: sample histological section for monkey KZ. Arrows indicate penetrations for muscimol injections which were marked at the end of experiments. *B*: line drawing of cerebellar nuclei (DN, dentate nucleus; IPN, interpositus nucleus; FN, fastigial nucleus) on which injection sites are indicated by circles. Injection sites in and around the dentate nucleus are numbered and labeled as shown (top right). *C*: database for muscimol injection experiments. Number of injections and the number of new and learned hypersets examined are shown for each site indicated in *B*. Note that the same learned hypersets were used repeatedly in different experiments.

**APPARATUS.** Our device for pressure injections was a stainless steel tube connected to a Hamilton syringe ( $10 \mu\text{l}$ ) by a polyethylene tubing. We used a stainless steel tube (ID: 0.10 mm, OD: 0.3 mm, length: 80 mm) with a sharp angle at the tip, to which a tungsten microelectrode (diameter: 0.25 mm, length: 80 mm, impedance: 0.4–2.0 M $\Omega$  measured at 1 kHz, Frederic Haer) was attached side by side with a quick-drying glue so that the tip of electrode protruded from the tip of injection tube by 0.2–0.3 mm. The injection tube was connected to the Hamilton syringe by a polyethylene tube (diameter: 0.3 mm).

**INJECTION PROCEDURE.** The injection tube and the Hamilton syringe first were filled with a silicon oil. Before an injection experiment, a small amount of muscimol solution was aspirated into the injection tube until the border between the silicon oil and the muscimol solution was visible in the polyethylene tube. The injection tube then was held by the micromanipulator and was inserted into the cerebellum. The depth of the injection was determined by the data obtained in the preceding recording sessions. In most experiments, we recorded neuronal activities with the microelectrode attached to the injection tube to confirm that the task-related neurons were recorded at the location of the injection. The muscimol solution was pressure-injected in several steps ( $0.2 \mu\text{l}$  for each step) with  $\geq 15 \text{ s}$  between steps. The total amount injected was 1–2  $\mu\text{l}$  for each site. A successful injection was indicated by

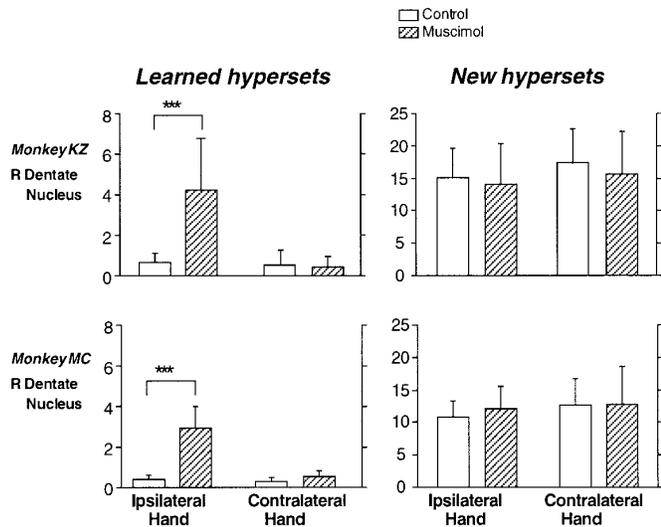


FIG. 3. Effects of muscimol injections in the dorsolateral part of the dentate nucleus on the number of errors. Number of errors before completing 10 successful trials (means  $\pm$  SD) is compared between the control experiments (saline injections; □) and muscimol injections (▨), separately for monkeys, kinds of hypersets (learned vs. new), and the hand used (ipsi- vs. contralateral to the injection). Number of errors for learned hypersets increased significantly when performed by the ipsilateral hand ( $***P < 0.001$ ) but not the contralateral hand ( $P > 0.05$ ). In contrast, the number of errors for new hypersets showed no change ( $P > 0.05$ ).

motions of the border between silicon oil and muscimol solution in the polyethylene tube and a silencing of neuronal activity as the injection started.

**Experimental procedures**

Before an injection, we had the monkey perform several learned and new hypersets using each hand to make sure that the monkey was well motivated and the performance was within the normal range. The postmuscimol data were obtained during a 150-min period (30–180 min after the injection). Experiments were performed in blocks, in each of which a particular hyperset was used

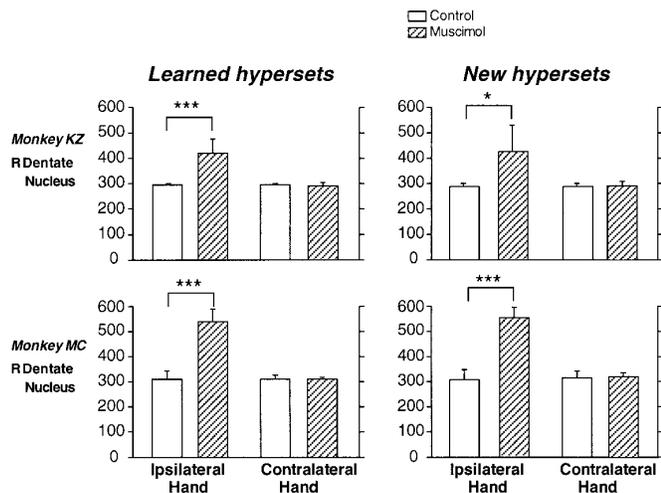


FIG. 4. Effects of muscimol injections in the dorsolateral part of the dentate nucleus on the movement time. Same format as in Fig. 3. Movement time for the ipsilateral hand increased both for the learned (left) and new (right) hypersets. Significance levels are indicated:  $***P < 0.001$ ;  $*P < 0.05$ .

repeatedly until the monkey completed 10 successful trials. The time spent for completing a block of experiment was  $\sim 3$  min for a learned hyperset and 4–6 min for a new hyperset. The time between blocks was  $\sim 2$  min. Thus we obtained data for 12–20 learned hypersets and 6–10 new hypersets after each muscimol injection. In addition, we examined 6–10 blocks of the simple reaction task. The three kinds of tasks were performed in blocks, each block consisting of two learned hypersets, one new hyperset, and one simple reaction task, in a random order. The same hand was used during a block, and the hand was changed in the next block. Data were obtained also on the next day that were used to confirm that the monkey’s performance recovered.

Control data were obtained also for a 150-min period when 1 or 2  $\mu$ l of normal saline was injected. Injection sites were restricted to the dentate nucleus. To exclude the possibility that the drug effect remained, we stopped making muscimol injections for  $\geq 6$  days before each experiment (muscimol injection or control experiment).

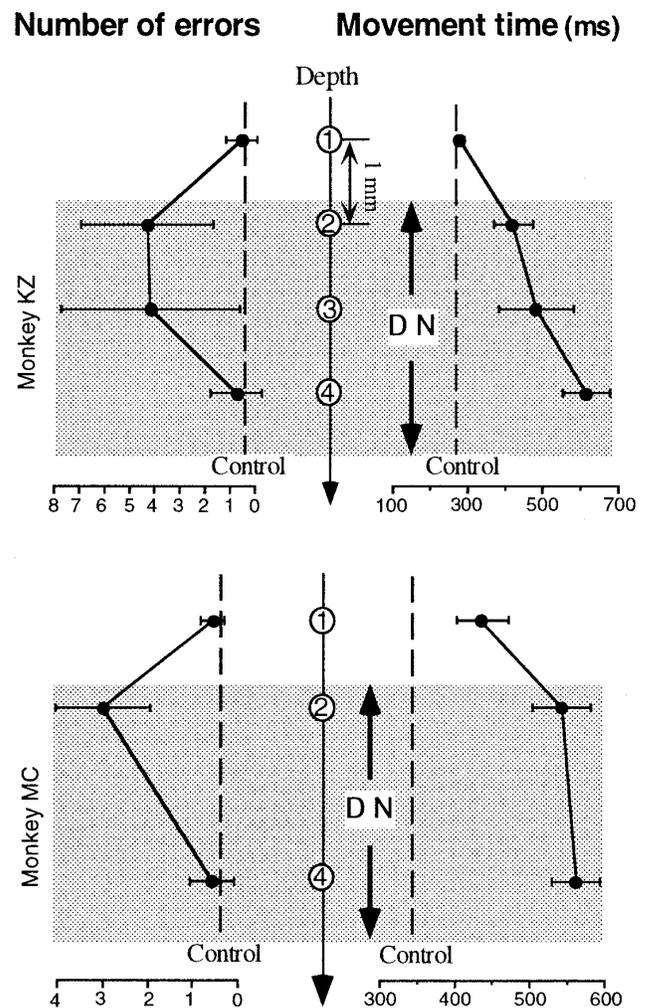


FIG. 5. Muscimol effects in the dentate nucleus changed with the depth of injection sites. Number of errors (left) and movement time (right; means  $\pm$  SD) are shown for each depth of injection through the dentate nucleus (indicated by the shadowed area, DN). Data were obtained by injections made through a guide tube for each monkey. Their locations are shown in Fig. 2 using the same symbols (numbered circles). Hatched lines show the mean control values obtained by saline injections. In both monkeys, the increase in the number of errors was confined in the dorsal and central parts of the dentate nucleus, whereas the increase in the movement time was more uniform.

*Data analysis*

The behavioral tasks as well as storage and display of data were controlled by a computer (PC 9801RA, NEC, Tokyo). The time and nature of task-related events (e.g., onset and offset of a LED target, pressing and releasing of buttons) were stored into an event file. Eye positions were digitized at 500 Hz and stored into an analog file continuously during each block of trials. Eye movements were recorded using the search coil method (Enzanshi Kogyo MEL-20U). In addition, the performance of the monkey was video-recorded.

We analyzed several parameters to assess the monkey's performance. A major parameter was the number of errors, or failed trials, before completing 10 successful trials. Only one type of error, sequence error, was counted in which the monkey pressed an illuminated button in wrong order. The number of errors to criterion was obtained for each block of experiment (i.e., for each hyperset performed).

We also measured the movement time and calculated the ratio of anticipatory saccades. The movement time was the time between the releasing of the first button to the pressing of the second button within a set; this value would reflect the speed of hand movements that would not be modified by the grade of sequence learning (see Fig. 4). The movement times were obtained for each successfully performed set, and then the values obtained in a block of experiment were averaged. For each block of the experiment, we also counted the number of anticipatory saccades, i.e., the saccades that started before the button illumination and reached the location of the first button (see Fig. 1). An increase in this value indicates the extent of long-term learning (Miyashita et al. 1996a). The maximum number of anticipatory saccades could be 40 (set 2-5, 10 successful trials).

Statistical comparisons (Mann-Whitney's *U*-test) were made for each of the above parameters between the postmuscimol values and the postsaline (control) values for each muscimol injection site. The number of samples corresponded to the number of blocks (or hypersets). The data obtained from different experiments were pooled if muscimol was injected at the same location repeatedly (see Fig. 2).

*Histology*

At the completion of the experiments or before we replaced the guide tube, we marked, by electrolytic lesions, the injection sites using the same coordinates through the guide tubes; the lesions were made by passing a DC current through the electrode (5  $\mu$ A, 200 s, electrode positive). *Monkey KZ* was anesthetized with an

overdose of pentobarbital sodium and perfused through the heart with 4% formalin. The brain was blocked and equilibrated with 30% sucrose. Frozen sections were cut at 50  $\mu$ m in planes parallel to the electrode penetrations so that tracks for injections were visible in single sections. The sections were stained with thionine. Locations of the guide tubes and electrolytic lesions were identified, based on which the injection sites were reconstructed (see Fig. 2A).

*MRI*

After the implantation of the guide tubes, we obtained MRIs for both monkeys (Hitachi AIRIS, 0.3 tesla). We confirmed in *monkey KZ* that the injection sites estimated on the MRIs nicely corresponded with the histologically identified microlesions. In *monkey MC*, the reconstruction of injection sites was based on the MRIs.

RESULTS

We made muscimol injections in different portions in the cerebellar nuclei, as shown in Fig. 2. In the earlier part of our study, we concentrated on the injections in the dentate nucleus. For this purpose, we inserted an injection pipette through an implanted guide tube that was aimed at the dentate nucleus. Muscimol was injected at different depths along the track, but repeatedly at each depth (see Fig. 2). We found that the muscimol injections in the dentate nucleus led to significant deficits in learned performances. In the later part of our study, we examined whether the muscimol effects were localized in the dentate nucleus. This was done by repositioning the guide tube to different portions of the dentate, interpositus, and fastigial nucleus. In the following, we will describe the results in two parts. First, we will show how muscimol in the dentate nucleus affected the learning/memory. Second, we will examine whether the muscimol effects varied depending on the locations in the cerebellar nuclei.

*Effects of dentate nucleus inactivation*

In this section, we concentrate on the results from muscimol injections in the dorsolateral part of the dentate nucleus (Fig. 2, *site 2*) as they showed a characteristic deficit in the performance of learned sequences. The data are based on

TABLE 1. *Effects of muscimol on number of errors*

Injection Site	Monkey	Learned Hypersets				New Hypersets			
		Ipsilateral Hand		Contralateral Hand		Ipsilateral Hand		Contralateral Hand	
		Control	Muscimol	Control	Muscimol	Control	Muscimol	Control	Muscimol
Dorsomedial DN	<i>KZ</i>	0.5	4.5 <sup>++</sup>	0.4	0.4	15.5	16.1	16.3	16.2
Dorsolateral DN	<i>KZ</i>	0.6	4.2 <sup>++</sup>	0.5	0.4	15.1	14.0	17.2	15.6
Dorsolateral DN	<i>MC</i>	0.4	3.0 <sup>++</sup>	0.3	0.5	11.0	12.0	12.6	12.8
Central DN	<i>KZ</i>	0.4	4.1 <sup>++</sup>	0.5	0.4	14.5	15.7	16.1	15.8
Lateral DN	<i>KZ</i>	0.5	0.8	0.5	0.3	16.3	16.5	16.4	14.5
Ventral DN	<i>KZ</i>	0.6	0.7	0.5	0.7	13.5	15.1	17.3	16.5
Ventral DN	<i>MC</i>	0.4	0.6	0.3	0.2	10.9	9.7	12.6	9.5
IPN	<i>KZ</i>	0.5	0.7	0.4	0.2	15.5	16.2	16.3	13.5
FN	<i>KZ</i>	0.5	0.5	0.5	0.8	16.3	15.0	16.4	13.0

The mean numbers of errors before completing 10 successful trials for learned and new hypersets. The data are shown separately for injection sites and monkeys. The injection sites correspond to those shown in Fig. 2. DN, dentate nucleus; IPN, interpositus nucleus; FN, fastigial nucleus. Significant differences between the control and post-muscimol data are indicated as <sup>++</sup> *P* < 0.001 (Mann-Whitney's *U* test). The same format applies to Table 2-4.

TABLE 2. *Effects of muscimol on movement time*

Injection Site	Monkey	Learned Hypersets				New Hypersets			
		Ipsilateral Hand		Contralateral Hand		Ipsilateral Hand		Contralateral Hand	
		Control	Muscimol	Control	Muscimol	Control	Muscimol	Control	Muscimol
Dorsomedial DN	KZ	294.0	563.4 <sup>++</sup>	310.3	288.8	291.3	524.2 <sup>++</sup>	310.2	298.4
Dorsolateral DN	KZ	298.2	417.7 <sup>++</sup>	297.7	296.0	290.2	419.3*	291.4	290.8
Dorsolateral DN	MC	308.0	540.6 <sup>++</sup>	309.0	308.7	305.4	564.1 <sup>++</sup>	310.0	316.0
Central DN	KZ	297.3	470.8 <sup>++</sup>	305.2	290.4	292.7	476.5 <sup>++</sup>	308.2	296.5
Lateral DN	KZ	285.7	384.4 <sup>++</sup>	301.0	297.2	285.3	403.6 <sup>++</sup>	300.9	297.6
Ventral DN	KZ	304.0	612.8 <sup>++</sup>	299.7	306.0	307.3	595.9 <sup>++</sup>	296.5	312.1
Ventral DN	MC	308.0	565.6 <sup>++</sup>	309.0	301.4	305.4	553.8 <sup>++</sup>	310.0	320.2
IPN	KZ	297.7	538.6 <sup>++</sup>	310.3	294.2	291.4	540.3 <sup>++</sup>	310.2	287.3
FN	KZ	301.1	374.2 <sup>+</sup>	285.7	310.9	300.9	420.4 <sup>+</sup>	285.3	335.4

Movement time in milliseconds. Significant differences between the control and postmuscimol data are indicated as \*  $P < 0.05$ ; +  $P < 0.01$ ; ++  $P < 0.001$  (Mann-Whitney's  $U$  test).

seven muscimol injections (4 in *monkey KZ*, 3 in *monkey MC*). The total number of hypersets examined was 95 (learned: 64, new: 31) for *monkey KZ* and 73 (learned: 49, new: 24) for *monkey MC*. We used the same learned hypersets repeatedly, but a given learned hyperset was used usually only once after each injection. New hypersets were generated each time, were used only once, and were never used again. We compared the postmuscimol with the control data that were obtained by saline injections.

**NUMBER OF ERRORS.** In the control condition, the number of errors was larger for new hypersets than for learned hypersets ( $P < 0.001$ ), reflecting a difference in the grade of learning. After muscimol injections into the dorsolateral part of the dentate nucleus, the number of errors for learned hypersets increased significantly in both monkeys (Fig. 3, *left*). This was true only when the hypersets were performed by the ipsilateral hand ( $P < 0.001$ ) but not by the contralateral hand ( $P > 0.05$ ). In contrast, the number of errors for new hypersets showed no change ( $P > 0.05$ ) (Fig. 3, *right*).

**MOVEMENT TIME.** In the control condition, the movement time was not different between learned hypersets and new hypersets ( $P > 0.05$ ), indicating that this parameter was not modified by the grade of learning (Fig. 4). After muscimol injections, the movement time increased significantly for learned as well as new hypersets, though there was no increase in the number of errors. The effects were restricted to the ipsilateral hand (learned hypersets:  $P < 0.001$  for 2 monkeys; new hypersets:  $P < 0.05$  for monkey KZ and  $P < 0.001$  for monkey MC), but not the contralateral hand ( $P > 0.05$ ).

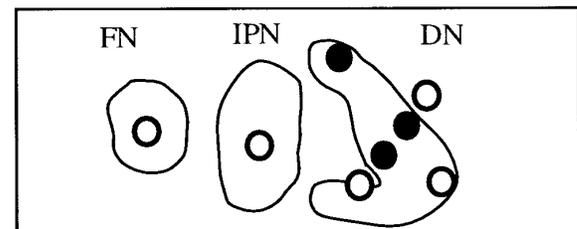
#### Functional localization in the cerebellar nuclei

The preceding results suggested that the dentate nucleus contributes to the long-term storage of motor skills. However, the dentate nucleus is likely to be composed of multiple functional zones (Middleton and Strick 1996). It was thus important to investigate which region in the dentate nucleus is related to the long-term storage of motor skills. Furthermore, the other cerebellar nuclei (interpositus and fastigial) also may contribute to such memory functions.

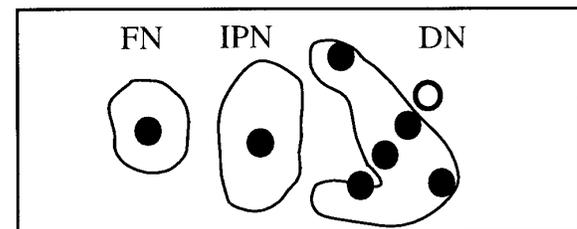
To answer these questions, we first changed the depths of injection sites in and around the dentate nucleus. The results obtained for learned hypersets performed by the ipsilateral

hand are shown in Fig. 5 for the two monkeys. The sites of injections are shown in Fig. 2 using the same symbols. We found that the number of errors and the movement time were affected differentially. In both monkeys, a significant increase in the number of errors resulted from muscimol injections in the dorsal and central parts of the dentate nucleus ( $P < 0.001$ , see Table 1); no change was observed by either injections just above the nucleus or injections in its ventral part ( $P > 0.05$ ). In contrast, the prolongation of the movement time was observed by both the dorsal and ventral injections ( $P < 0.001$ , see Table 2) but tended to be more pronounced in the ventral part. These results suggested that the memory-related function may be localized in the dorsal and central parts of the dentate nucleus, whereas

#### Number of Errors



#### Movement Time



- Effective sites
- Non-effective sites

FIG. 6. Summary diagram showing the distribution of muscimol effects in the cerebellar nuclei. Number of errors increased after the injections in the dorsomedial, dorsolateral, and central parts of the dentate nucleus (DN). Movement time increased after all injections except for the injection above the dentate nucleus.

TABLE 3. *Effects of muscimol on number of anticipatory saccades*

Injection Site	Monkey	Learned Hypersets				New Hypersets			
		Ipsilateral Hand		Contralateral Hand		Ipsilateral Hand		Contralateral Hand	
		Control	Muscimol	Control	Muscimol	Control	Muscimol	Control	Muscimol
Dorsomedial DN	KZ	17.0	11.9 <sup>+</sup>	16.9	16.2	2.4	4.5	7.0	5.2
Dorsolateral DN	KZ	19.2	8.8 <sup>++</sup>	18.5	11.5 <sup>+</sup>	2.7	4.2	4.1	3.3
Dorsolateral DN	MC	10.9	7.5 <sup>+</sup>	10.0	11.8	4.4	3.7	3.8	7.3
Central DN	KZ	17.3	10.1 <sup>+</sup>	16.5	11.2 <sup>+</sup>	2.8	4.3	6.1	4.6
Lateral DN	KZ	17.7	15.8	18.4	19.1	2.6	4.0	3.9	1.5
Ventral DN	KZ	18.8	15.4	17.1	15.6	3.4	2.0	2.4	1.0
Ventral DN	MC	10.9	7.4	10.0	9.4	4.4	2.7	3.8	2.0
IPN	KZ	16.9	11.0 <sup>+</sup>	16.9	18.2	2.4	4.7	7.0	4.0
FN	KZ	18.4	9.7	17.7	11.5	5.9	3.3	2.6	0.7

Significant differences between the control and postmuscimol data are indicated as <sup>+</sup>  $P < 0.01$ ; <sup>++</sup>  $P < 0.001$  (Mann-Whitney's *U* test).

the movement-related function is distributed more widely in the nucleus. We also calculated the correlation between the number of errors and the movement time for all learned hypersets examined after all muscimol injections along the penetrations (Fig. 5), and there was no correlation for both monkeys ( $P > 0.05$ , Spearman rank correlation test).

The localization of the memory function in the dorsal and central part of the dentate nucleus was confirmed by a wider survey of the cerebellar nuclei, including the interpositus nucleus (IPN) and fastigial nucleus (FN) (Fig. 2). The results are shown in Table 1–4 and are summarized in Fig. 6.

A significant increase in the number of errors was observed only by the muscimol injections in the dorsolateral, dorsomedial, and central parts of the dentate nucleus (Table 1). Again, the effects were restricted to the learned hypersets that were performed by the ipsilateral hand. Injections in the lateral and ventral parts of the dentate nucleus and the interpositus and fastigial nuclei led to no change in the number of errors.

In contrast, the movement time increased by muscimol injections at all sites (Table 2), except for the site above the dentate nucleus (Fig. 6). The effect was present for both learned and new hypersets but was restricted to the ipsilateral hand.

*Effects on saccadic eye movements*

A skillful performance of the 2 × 5 task was characterized by the occurrence of anticipatory saccades. After long-term

practice of a hyperset, the monkey makes a saccade to the next position of the target before it is illuminated (see Fig. 1) (Miyashita et al. 1996a). Such anticipatory saccades became infrequent after muscimol injections in the cerebellar nuclei (Table 3).

The number of anticipatory saccades for learned hypersets decreased when muscimol was injected into the dorsolateral, dorsomedial, and central parts of the dentate nucleus and the interpositus nucleus (Table 3). Interestingly, the effects tended to be greater when the ipsilateral hand was used, especially for the dorsomedial dentate nucleus. Anticipatory saccades tended to be less frequent after fastigial nucleus injections during the use of both the ipsilateral and contralateral hands, though not significantly. The number of the anticipatory saccades for new hypersets showed no significant change ( $P > 0.05$ ).

These results raised the possibility that anticipatory saccades became less frequent because the initiation of saccades became deficient. This hypothesis was not supported, however, as the latency of saccades during the simple reaction task showed a different pattern of changes after muscimol injections (Table 4). For example, after the injections in the dorsolateral part of the dentate nucleus, the saccade latency showed no change whereas the anticipatory saccade became less frequent. However, we cannot exclude the possibility that changes in other saccade parameters such as saccade velocity or accuracy may have contributed to the decrease in the ratio of anticipatory saccades. The saccade latency

TABLE 4. *Effects of muscimol on saccade latency*

Injection Site	Monkey	Ipsilateral Hand		Contralateral Hand	
		Control	Muscimol	Control	Muscimol
Dorsomedial DN	KZ	129.3	145.4 <sup>+</sup>	128.4	133.0
Dorsolateral DN	KZ	125.4	124.5	126.7	121.2
Dorsolateral DN	MC	129.2	123.5	124.1	127.6
Central DN	KZ	128.8	126.5	127.2	126.8
Lateral DN	KZ	126.8	133.3	126.6	133.0
Ventral DN	KZ	128.4	130.5	126.8	124.5
Ventral DN	MC	129.2	128.5	124.1	127.6
IPN	KZ	129.3	160.7 <sup>+</sup>	128.4	144.1
FN	KZ	126.6	149.9 <sup>++</sup>	126.8	133.9

Latencies are in milliseconds. Significant differences between the control and postmuscimol data are indicated as: <sup>+</sup>  $P < 0.01$ ; <sup>++</sup>  $P < 0.001$  (Mann-Whitney's *U* test).

increased after injections in the dorsomedial part of the dentate nucleus as well as the interpositus and fastigial nucleus and only when the ipsilateral hand was used.

## DISCUSSION

### *Role of dentate nucleus in motor skill*

The purpose of this study was to examine whether the cerebellar nuclei are involved in procedural memory. Two main results may be stressed. First, after the reversible inactivation of the dorsal and central regions of the dentate nucleus by injecting muscimol, the number of errors increased significantly for learned sequences, not new sequences, only when the hand ipsilateral to the muscimol injection was used. Second, the number of errors showed no significant change after muscimol injections into the ventral or lateral dentate nucleus, interpositus nucleus, or fastigial nucleus. These results suggest that the dorsal and central regions of the dentate nucleus are related to the storage and/or retrieval of long-term procedural memory rather than its acquisition.

A salient feature in our study was that the effects of muscimol on learning/memory were selective for the ipsilateral hand, in the same way as cerebellar motor symptoms. This is consistent with the result of a behavioral study when, after learning a sequence extensively using one hand, the monkey was required to use the other hand, the number of errors increased significantly (Rand et al. 1998). These results suggest that, with a long-term practice, the monkeys depended less on the explicit knowledge for button order (which is likely to be available to both hands) and more on the implicit motor skill (specific to the hand), in which the dentate nucleus plays a preferential role.

### *Functional subregions in the dentate nucleus*

The effects of muscimol injections on learning/memory were localized in the dorsal and central parts of the dentate nucleus. We suspect, however, that the actual locus might be more localized than what appears from Fig. 5 or 6. For example, the *injection site 1 or 4* in Fig. 5 produced virtually no effect, but in this case, the *site 2 or 3* may well have been infused with muscimol by diffusion; then, the effective site may be somewhere between *site 2* and *3*. Alternatively, the memory-related zone might be larger than what appears from Fig. 5 or 6; if a behavioral change occurs only after inactivation of a considerable portion of the memory-related zone, muscimol injection at its fringe area would not lead to a significant change in the number of errors.

Middleton and Strick (1996) and Wiesendanger and Wiesendanger (1985) have shown that the dentate nucleus consists of subregions that project, through the thalamus, to different parts of the frontal lobe. Although the dorsal part projects to the primary motor cortex (M1), the lateral part projects to the premotor and supplementary motor area, and the ventral part projects to the prefrontal cortex and presupplementary motor area. The suggested relationship of the dorsal dentate nucleus with M1 is consistent with the unilateral nature of the muscimol effects because the M1 controls the contralateral hand almost exclusively. The neural substrates for long-term motor skills thus would involve the M1, as suggested previously (Asanuma and Pavlides 1997;

Kleim et al. 1996, 1997; Nudo et al. 1996; Salmon and Butters 1995), and its connections with the cerebellum (Kleim et al. 1997).

In our laboratory, Miyashita et al. (1996b, 1997) in monkeys and Hikosaka et al. (1996b) in humans have shown that the presupplementary motor area is related to acquisition of new motor sequences. These results, together with the anatomic findings, suggested that the inactivation of the ventral part of the dentate nucleus leads to deficits in acquisition of new sequences; however, this is not what we found. Our failure could just be technical in that bilateral or more extensive inactivation would be necessary to produce deficits in acquisition of new sequences. An alternative possibility is that the cerebral cortex (Miyashita et al. 1996b; Sakai et al. 1998) and basal ganglia (Miyachi et al. 1997) are sufficient for acquisition of new sequences, even though the cerebellum may play a role in acquisition.

### *Eye-hand coordination as an expression of cerebellar-dependent motor skill*

A high degree of motor skill in the  $2 \times 5$  task requires anticipatory saccades (Miyashita et al. 1996a). After practicing a hyperset for  $>10$  days, the eye starts moving toward the location of the target before it is illuminated, which greatly improves accuracy and therefore performance. We showed that the ratio of such anticipatory saccades decreased significantly after the inactivation of the dentate nucleus (dorsal and central parts) and the interpositus nucleus. These results suggest that the dentate nucleus contributes to the expression of long-term procedural memory.

Interestingly, the decrease in the ratio of anticipatory saccades was more evident when the hand ipsilateral to the inactivation was used. This finding, which may appear peculiar, is consistent with the results from an earlier study (Miyashita et al. 1996a) in which the monkeys were asked to use the hand that had not been used for practice. The ratio of anticipatory saccades decreased while the number of errors increased. These results are consistent with the hypothesized role of the dentate nucleus in memory functions and further suggest that the memory engram is more accessible to the hand used during practice.

It actually has been suggested that the cerebellum is crucial for the eye-hand coordination. The coordinated eye-hand movements, compared with each movement alone, improve the accuracy of these movement in normal subjects but degrade the accuracy in cerebellar patients (Van Donkelaar and Lee 1994). After a lesion of the dentate nucleus in baboons, the eye-hand coordination deteriorated when the hand ipsilateral to the lesion was used (Vercher and Gauthier 1988).

One might argue that the paucity of anticipatory saccades was due to more general deficits of saccades, as would be suggested by lesion studies (Büttner et al. 1994; Ritchie 1976; Vilis and Hore 1981) and single-unit studies (Fuchs et al. 1993; Keller 1989; MacKay 1988; Ohtsuka and Noda 1991; Van Kan et al. 1993). Although the saccade latency did increase by the inactivation of the cerebellar nuclei (Table 4), it cannot account for the paucity of anticipatory saccades. However, changes in other saccade parameters might have contributed to the paucity of anticipatory saccades.

*Sequential versus parallel processing during visuomotor sequence learning*

Studies in our laboratory using the  $2 \times 5$  task have shown that different regions in the cerebral cortex and the basal ganglia contribute either to acquisition of new motor sequences or to long-term storage of visuomotor sequences. By local inactivation with muscimol, Miyachi et al. (1997) showed that the anterior striatum and the posterior striatum (putamen) are related to the acquisition of new sequences and the memory storage, respectively. Using single-unit recording and muscimol injections, Miyashita et al. (1996b, 1997) showed that the presupplementary motor area (pre-SMA) in the medial premotor cortex was crucial for the acquisition of new sequences, especially the acquisition of sequential knowledge (as assessed by the number of errors). The supplementary motor area (SMA) also contributed to the acquisition of new sequences but in terms of procedural skill (as assessed by the reaction time) rather than knowledge. The role of the pre-SMA in the acquisition of new sequences was confirmed in humans by a study using functional MRI (Hikosaka et al. 1996b). Sakai et al. (1998) further showed learning-related transition of activation from the frontal to parietal cortical regions. Frontal cortical regions, the pre-SMA together with the dorsolateral prefrontal cortex, were activated in the early phase of learning, whereas the parietal cortex, medially in the precuneus and laterally in the intraparietal sulcus, was activated in the intermediate and advanced stages. These cortical activations, however, do not reflect the long-term memory storage because they tended to decrease eventually as the sequential movements became skillful and automatic.

The present study showed that the cerebellar dentate nucleus is related to the skill for sequential movements that is acquired by a long-term practice. However, the monkeys' performance in the learned sequences after the inactivation of the dentate nucleus, though worse than the control level, was still better than the performance in new sequences. Therefore it seems unlikely that the dentate nucleus is the only area related to the skill for sequential movements; other brain areas, including the posterior putamen (Miyachi et al. 1997), also would contribute to this function.

Our conclusion may appear inconsistent with recent imaging studies using positron emission tomography and functional magnetic resonance imaging showing that the cerebellum is activated during motor and nonmotor learning, especially its early phase (Flament et al. 1996; Jenkins et al. 1994; Kim et al. 1994; Raichle et al. 1994; Seitz et al. 1994). However, these findings may not necessarily indicate that the cerebellum is needed in the early phase of learning. On the other hand, our results does not indicate that there was no cerebellar activation in the early phase of learning. We propose that the seemingly contradictory results may be accounted for by parallel organization of learning-related functions. According to this hypothesis, the cerebellum starts working to acquire the motor skills from the beginning (as imaging studies suggest), but a loss of its function would not result in a significant retardation of learning (as our results suggest), presumably because the procedural knowledge is maintained by the cortex-basal ganglia mechanism. Only after a long-term practice would the loss of the cerebellar

function affect the performance, presumably because the subject is now less dependent on the cortex-basal ganglia mechanism. To summarize, during the long-term course of visuomotor sequence learning, the cerebral cortex, basal ganglia, and cerebellum initially may work in parallel but in unique manners, but eventually the majority of the visuomotor sequence skill shifts toward the cerebellum, perhaps in addition to the posterior striatum (Miyachi et al. 1997). A similar shift of learning functions was suggested by Shadmehr and Holcomb (1997) for a motor skill learning in humans.

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