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MEMORY TRACE OF MOTOR LEARNING SHIFTS TRANSSYNAPTICALLY FROM CEREBELLAR CORTEX TO NUCLEI FOR CONSOLIDATION

F. SHUTOH,[a,e] M. OHKI,[a,b] H. KITAZAWA,[a] S. ITOHARA[c] AND S. NAGAO[a,d]*

[a]*Laboratory for Motor Learning Control, RIKEN Brain Science Institute, Hirosawa 2-1, Wako, Saitama 351-0198, Japan*

[b]*Department of Otorhinolaryngology, Graduate School of Medicine, University of Tokyo, Hongo 7-3-1, Bunkyo-Ku, Tokyo 113-0033, Japan*

[c]*Laboratory for Behavioral Genetics, RIKEN Brain Science Institute, Hirosawa 2-1, Wako, Saitama 351-0198, Japan*

[d]*SORST, Japan Science and Technology Corporation, Honcho 4-1-8, Kawaguchi, Saitama 331-0012, Japan*

[e]*Laboratory of Neuroendocrinology, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tennodai 1-1-1, Tsukuba, Ibaraki 305-8574, Japan*

Abstract—Adaptation of ocular reflexes is a prototype of motor learning. While the cerebellum is acknowledged as the critical site for motor learning, the functional differences between the cerebellar cortex and nuclei in motor memory formation are not precisely known. Two different views are proposed: one that the memory is formed within the cerebellar flocculus, and the other that the memory is formed within vestibular nuclei. Here we developed a new paradigm of long-term adaptation of mouse horizontal optokinetic response eye movements and examined the location of its memory trace. We also tested the role of flocculus and inferior olive in long-term adaptation by chronic lesion experiments. Reversible bilateral flocculus shutdown with local application of 0.5 μl–5% lidocaine extinguished the memory trace of day-long adaptation, while it very little affected the memory trace of week-long adaptation. The responsiveness of vestibular nuclei after week-long adaptation was examined by measuring the extracellular field responses to the electrical stimulation of vestibular nerve under trichloroacetaldehyde anesthesia. The amplitudes and slopes of evoked monosynaptic field response (N_1) of week-long adapted mice were enhanced around the medial vestibular nucleus compared with those of control mice. Chronic flocculus or inferior olive lesions abolished both day and week-long adaptations. These results suggest that the functional memory trace of short-term adaptation is formed initially within the cerebellar cortex, and later transferred to vestibular nuclei to be consolidated to a long-term memory. Both day and week-long adaptations were markedly depressed when neural nitric oxide was pharmacologically blocked locally and when neuronal nitric oxide synthase was ablated by gene knockout, suggesting that cerebellar long-term depression underlies both acquisition and consolidation of motor memory. © 2005 Published by Elsevier Ltd on behalf of IBRO.

Key words: adaptation, optokinetic response, vestibulo-ocular reflex, long-term depression, lidocaine chloride, nitric oxide.

Long-term training is necessary to acquire skilled motor movement. While the cerebellum is supposed to be the critical site for motor learning, its precise role in the acquisition and maintenance of motor memory is a controversial topic (Melvill-Jones, 2000; Ito, 1984; du Lac et al., 1995). Two opposing views have been suggested for the role of the cerebellum. One suggests that motor learning may be induced by long-term depression (LTD) of parallel fiber-Purkinje cell synapses with the associated motor memory stored within the cerebellar cortex (Ito, 1989, 1998). The other rejects such a role for the cerebellum, suggesting instead that motor memory is formed in the vestibular or cerebellar nuclear neurons utilizing signals mediated by the cerebellum (Lisberger and Sejnowski, 1992). Recently, a third view is proposed that multiple plasticity mechanisms or neural sites may be involved in cerebellum-dependent motor learning (van Alphen and De Zeeuw, 2002; Blazquez et al., 2004; Boyden et al., 2004). To help to resolve these debates, we developed a new ocular reflex adaptation paradigm to simultaneously and quantitatively evaluate short and long-term motor learning using mice, and examine the locations of their memory traces with pharmacological and electrophysiological experiments. The role of the cerebellum and LTD in short and long-term motor learning was evaluated using lesion, pharmacological, and gene knockout experiments in mice. We found that LTD plays essential roles in the induction of motor learning within the cerebellar cortex, and in the induction of neural substrate of memory consolidation located within the vestibular nuclei.

EXPERIMENTAL PROCEDURES

Animal preparation

The experimental protocols followed the principles of laboratory animal care (U.S. National Institutes of Health publication No. 86-23, 1978) and were approved by the management committee of RIKEN Brain Science Institute. C57BL/6j mice obtained from Japan Clea (Tokyo, Japan) were used in all experiments except for those using gene knockout mice. All efforts were made to

*Correspondence to: S. Nagao, Laboratory for Motor Learning Control, RIKEN Brain Science Institute, Hirosawa 2-1, Wako, Saitama 351-0198, Japan. Tel: +81-48-467-9794; fax: +81-48-467-9793.
E-mail address: nagaos@brain.riken.jp (S. Nagao).

Abbreviations: D-NMMA, N^G-monomethyl-D-arginine; EPSP, excitatory post-synaptic potential; FITC, fluorescein isothiocyanate hydrochloride; HOKR, horizontal optokinetic response; HVOR, horizontal vestibulo-ocular reflex; L-NMMA, N^G-monomethyl-L-arginine; LTD, long-term depression; LTP, long-term potentiation; nNOS, neural nitric synthase; PBS, phosphate-buffered saline.

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minimize the number of mice used and their suffering. The mice lacking neural nitric synthase (*nNOS*) (B6, 129S-Nos1^{tm1PlH}) were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). The *nNOS*$^{-/-}$ mutants were backcrossed with C57BL/6j strain for two generations, and heterozygotes were intercrossed to obtain mutation homozygotes. Wild-type littermates were used as controls. Primers used for PCR-aided genotyping were 5′-AAACTCT-GCAGCCGGTTCTT-3′ and 5′-CCTGTCTACTGCTAATGGCT-3′ for wild-type allele and 5′-CTTGGGTGGAGAGGCTATTC-3′ and 5′-AGGTGAGATGACGGGAGATC-3′ for mutant allele (neo gene cassette). PCR cycles were run at 96 °C for 1 min, 60 °C for 1 min, and 72 °C for 2 min for 35 cycles. Mice used in the present study were approximately 3 months old.

Eye movement experiments

Under pentobarbital anesthesia (Somnopentyl; Schering Plough, Kenilworth, NJ, USA; 60 mg kg^{-1} body weight) and aseptic conditions, a platform for head fixation was made on the cranial bone using four small screws and one long bolt by synthetic resin. Small holes were made on the cranial bones over the paraflocculars lobe bilaterally. The mouse was mounted on the turntable surrounded by a checked-pattern screen, with its head fixed and its body loosely restrained in a plastic cylinder. Eye movements were recorded with an infrared TV camera for real-time recording (Katoh et al., 1998; Shutoh et al., 2002). The frontal view of the right eye was monitored using a CCD TV camera (SSC-M350; Sony, Tokyo, Japan) through a cold mirror. The pupil of the right eye was illuminated by an infrared-light (wavelength, 900 nm) -emitting diode and displayed on a 12-inch TV monitor (magnification, 55×). The area of the pupil was determined from the difference in brightness between the pupil and the iris. The real-time position of the eye was measured by calculating the central position of the left and right margins of the pupil at 50 Hz using a position-analyzing system (C-1170; Hamamatsu Photonics, Hamamatsu, Japan) and stored on a personal computer. Pupil size was kept constant either by exposing them to white noise sounds during horizontal optokinetic response (HOKR) to maintain alertness, or with drops of pilocarpine chloride (Sigma) solution applied locally during horizontal vestibulo-ocular reflex (HVOR). The mean effective diameter of the mouse eyeball was estimated to be 2.3 mm (Shutoh et al., 2002). The spatial resolution of TV camera system was 0.25°. HOKR was tested by sinusoidal oscillation of the checked-pattern screen (check size, 4°) by 5–20° (peak-to-peak) at 0.11–0.17 Hz (maximum screen velocity, 2.6–10.5°/s) in light. HVOR was tested by sinusoidal oscillation of the turntable in the horizontal plane by 10° (peak-to-peak) at 0.11–0.5 Hz in the dark, as reported previously (Katoh et al., 1998; Shutoh et al., 2003). Over 10 cycles of the evoked eye movements, free from artifacts due to blinks and saccades, were selected for averaging. No correction programs were used to delete artifacts. Mean amplitudes and phases were calculated by a modified Fourier analysis (Jastreboff, 1979). The gain of eye movement was defined as the ratio of the peak-to-peak amplitude of eye movements to that of the screen or turntable oscillation. The phase was defined as 0° when the peak of eye movement matched the peak of screen oscillation in HOKR, and when peak of eye movement was opposite to the peak of turntable oscillation in HVOR. Short-term adaptations of HOKR were examined by exposing a mouse to 1 h sustained screen oscillations at 15° and 0.17 Hz (maximum screen velocity, 7.9°/s), and at 10° and 0.11 Hz (maximum screen velocity, 3.5°/s) in light. Long-term adaptations of HOKR were examined by successively giving the trainings of short-term adaptations over 4–5 days. During long-term trainings, the mice were kept in darkness except for training sessions. Four mice were kept in cages under normal light conditions (12-h light/dark) after the end of 5-day optokinetic training, and HOKR was tested every 3–4 days for 2–3 weeks to see recovery from long-term HOKR adaptation.

Pharmacological experiments

Effects of flocculus shutdowns were examined in 18 mice. Lidocaine (0.5 μl, 5%) dissolved in standard Ringer's solution, or the same amount of control Ringer's solution (Nagao and Kitazawa, 2003), was manually injected into the bilateral flocculi using two microsyringes (80135, Hamilton, Reno, NV, USA) mounted on standard micromanipulators for 20 min at the end of the trainings on the 4th day, or after 24 h. HOKR was examined 30 min later or every 30 min for 1.5 h. The same amount of 5% lidocaine was also injected into the bilateral flocculi in naïve untrained mice. The concentration and volume of lidocaine was determined by the effects of microstimulation of flocculus using another five mice. We inserted glass microelectrodes containing 2 M NaCl solution (tip diameter, 2–4 μm; resistance, 0.5–1 MΩ) into the paraflocculus–flocculus complex 30–60 min after lidocaine injections. We delivered negative train pulses (frequency, 333 Hz; width, 0.15 ms; strength <50 μA; pulse number, 40; train frequency, 0.25 Hz) via the microelectrode every 0.2 mm from the surface to a depth of 1.0 mm, and observed evoked eye movements using a TV-camera system.

Effects of nNOS blockers were tested in six mice by locally injecting 0.5 μl of 20 mM N^G-monomethyl-L-arginine (L-NMMA, Sigma, St. Louis, MO, USA), dissolved in phosphate-buffered saline (PBS), into the bilateral flocculi in the same manner as lidocaine 1 h before the start of training on the 1st and 3rd days. As control, the same amount of N^G-monomethyl-D-arginine (D-NMMA, Sigma) was injected similarly into the bilateral flocculi in another five mice. In order to estimate the extent of drug diffusions, 0.5 μl of 1% fluorescein isothiocyanate hydrochloride (FITC, Research Organics, Cleveland, OH, USA) or 2% Alcien Blue (Sigma) dissolved in saline was injected similarly into the bilateral flocculi in 10 mice, and 3 h or 3 days later they were perfused with PBS and 4% paraformaldehyde under general anesthesia. Coronal sections (50 μm thick) were prepared for histological analyses.

Electrophysiological experiments

Nine long-term trained and 15 naïve control mice were used for experiments in the blind condition. Under general anesthesia (trichloroacetaldehyde monohydrate, Wako, Osaka, Japan, 10 mg kg^{-1}body weight) and aseptic conditions, mice were fixed with a standard stereotaxic apparatus. Body temperature was kept constant by a heating-pad. Their left temporal bones (auditory bulla) were opened, and two small holes (diameter, 100 μm) were opened on the lateral semicircular canal at 1 mm from the lateral crista ampullaris, and the posterior semicircular canal at 1.6 mm from the posterior crista ampullaris by a surgical drill. A bipolar stimulating microelectrode made of 0.02 mm polyimide-coated stainless wires (California Fine Wire Inc., CA, USA) was inserted into the semicircular canals (cathodal tip for horizontal canal, and anodal tip for posterior canal) under microscopy. After the bones covering the left cerebellum were removed, the cerebellar vermal-hemispheric lobe and lower part of the tectum were aspirated to expose the brainstem. In order to stabilize recording, surface of exposed brainstem was covered with white Vaseline dissolved in warmed liquid paraffin. Glass microelectrodes containing 2 M NaCl solution (resistance, 0.8–1.2 MΩ) attached with a standard micromanipulator were inserted systematically 0.5–1.75 mm from the midline in the medio-lateral direction and 0–1 mm from the rostral end of superior vestibular nucleus in the anterior–posterior direction at the interval of 250 μm. Extracellular field potentials evoked by electrical stimulation of vestibular nerve with monophasic negative (cathode at the lateral semicircular canal and anode at the posterior semicir-