The Role of Endogenous Neurogenesis in Functional Recovery and Motor Map Reorganization Induced by Rehabilitative Therapy after Stroke in Rats

Takashi Shiromoto, MD,*,† Naohiko Okabe, PhD,* Feng Lu, PhD,* Emi Maruyama-Nakamura, PhD,* Naoyuki Himi, PhD,* Kazuhiko Narita, PhD,* Yoshiki Yagita, MD,† Kazumi Kimura, MD,‡ and Osamu Miyamoto, MD*

Background and Objective: Endogenous neurogenesis is associated with functional recovery after stroke, but the roles it plays in such recovery processes are unknown. This study aims to clarify the roles of endogenous neurogenesis in functional recovery and motor map reorganization induced by rehabilitative therapy after stroke by using a rat model of cerebral ischemia (CI). *Methods:* Ischemia was induced via photothrombosis in the caudal forelimb area of the rat cortex. First, we examined the effect of rehabilitative therapy on functional recovery and motor map reorganization, using the skilled forelimb reaching test and intracortical microstimulation. Next, using the same approaches, we examined how motor map reorganization changed when endogenous neurogenesis after stroke was inhibited by cytosine-β-D-arabinofuranoside (Ara-C). *Results:* Rehabilitative therapy for 4 weeks after the induction of stroke significantly improved functional recovery and expanded the rostral forelimb area (RFA). Intraventricular Ara-C administration for 4-10 days after stroke significantly suppressed endogenous neurogenesis compared to vehicle, but did not appear to influence non-neural cells (e.g., microglia, astrocytes, and vascular endothelial cells). Suppressing endogenous neurogenesis via Ara-C administration significantly inhibited (~50% less than vehicle) functional recovery and RFA expansion (~33% of vehicle) induced by rehabilitative therapy after CI. *Conclusions:* After CI, inhibition of endogenous neurogenesis suppressed both the functional and anatomical markers of rehabilitative therapy. These results suggest that endogenous neurogenesis contributes to functional recovery after CI related to rehabilitative therapy, possibly through its promotion of motor map reorganization, although other additional roles cannot be ruled out.
Key Words: Cerebral ischemia—endogenous neurogenesis—motor map reorganization—motor recovery—rehabilitative therapy—cytosine-β-D-arabinofuranoside (Ara-C).

From the *Second Department of Physiology, Kawasaki Medical School, Kurashiki City, Okayama, Japan; †Department of Stroke Medicine, Kawasaki Medical School, Kurashiki City, Okayama, Japan; and ‡Department of Neurological Science, Graduate School of Medicine, Nippon Medical School, Tokyo, Japan.

Received May 13, 2016; revision received August 31, 2016; accepted September 11, 2016.

This work was supported by the JSPS KAKENHI Grant-in-Aid for Scientific Research (B), Grant Number 24700572.

Address correspondence to Naohiko Okabe, PhD, Second Department of Physiology, Kawasaki Medical School, 577 Matsushima, Kurashiki City, Okayama 701-0192, Japan. E-mail: n-okabe@med.kawasaki-m.ac.jp.

1052-3057/$ - see front matter

http://dx.doi.org/10.1016/j.jstrokecerebrovasdis.2016.09.016

Introduction

Stroke is a common cause of severe disability for which functional recovery is frequently limited.[1,2] Currently, intravenous tissue plasminogen activator[3,4] and endovascular treatment[5,6] are the only treatments that improve the clinical outcome of patients with acute ischemic stroke, but their effectiveness is limited by their narrow therapeutic time window.[7] Over the long term, 25%-74% of patients have to rely on human assistance for basic activities of daily living such as feeding, self-care, and mobility.[8] To improve the long-term prognosis in stroke, it is important to clarify the mechanisms of functional recovery that have not yet been identified or completely understood.

For the many stroke survivors unable to benefit from drug therapy, rehabilitative therapy can still promote the recovery of motor deficits.[9] The mechanisms underlying this recovery are unclear but appear to be related to cortical motor map reorganization.[10,11] In humans, noninvasive imaging data indicate that changes in cortical motor map topography are relevant to functional recovery following rehabilitative therapy.[12] In several other reports, endogenous neurogenesis in the subventricular zone (SVZ) contributes to the functional recovery after stroke. SVZ-derived, newly born neural stem cells migrate to areas of cerebral ischemia (CI), where they may contribute to postischemic repair[13-15] or the enhancement of endogenous neurogenesis by rehabilitative therapy.[16,17] Notably, inhibiting endogenous neurogenesis was found to worsen the functional outcome in mice after CI.[18,19] Given these data, we hypothesized that the inhibition of endogenous neurogenesis would decrease cerebral plasticity, as represented by motor map reorganization, and lead to the suppression of functional recovery in cases of ischemic stroke.

The aim of the present experiment was to investigate this hypothesis by analyzing the role of endogenous neurogenesis in functional recovery and motor map reorganization induced by rehabilitative therapy after CI. We analyzed the effects of rehabilitative therapy in rats after CI localized to the caudal forelimb area (CFA) of the cortex, assessing functional recovery and motor map reorganization with and without pharmacological inhibition of endogenous neurogenesis.

Methods

Animals

A total of 121 adult, male, Fisher 344 rats (7-8 week old, 160-180 g; CLEA Japan Inc., Tokyo, Japan) were used for all experiments. The rats were housed within a temperature-controlled vivarium on a 12-hour : 12-hour light : dark cycle. Food intake was moderately restricted throughout the study to maintain body weight at 80% of the ad libitum weight, but water was freely available. All experimental procedures were in accordance with National Institutes of Health regulations and were approved by the Animal Research Committee of Kawasaki Medical School.

Induction of Photothrombotic Ischemia

CI was induced via photothrombosis.[20] The rats were anesthetized with an intraperitoneal injection of ketamine hydrochloride (48 mg/kg)/xylazine hydrochloride (4.8 mg/kg). A bone window was created above the dominant CFA (3.5 mm lateral from the bregma, 3-mm diameter). A green (532 nm) light source attached to a 10× objective, providing a 2-mm-diameter illumination spot, was stereotactically centered on the bone window. The brain was illuminated for 15 minutes. During the first minutes of illumination, rose bengal (60 mg/kg) was injected via a tail vein catheter to stimulate thrombosis. Sham-operated rats underwent the same experimental procedures as described above but without the infusion of rose bengal.

Skilled Forelimb Reaching Task

The rats received a food tray task to habituate them to using the forelimb for 1 week, and 4 weeks of pretraining in the standard skilled forelimb reaching task.[21] Rats were placed within a Plexiglas reaching box (25 cm high × 10 cm wide × 25 cm long), with a tall narrow window (25 cm high × 1 cm wide) located in the middle of the 10-cm-wide wall. A horizontal plastic shelf (10 cm wide × 3 cm long) was mounted 3 cm from the floor on the front of the box. The rats reached through the window for a distance of 1.5 cm to retrieve a single food pellet held in shallow indentations on the shelf. A "trial" was defined when the pellet was placed on the shelf, after which the rat either consumed or missed reaching it. If a rat consumed a pellet without missing, knocking, or dropping it on the initial limb advance, the movement was scored as a "hit." A successful reaching score was calculated as follows: motor performance = (number of hits/25 trials) × 100. One session consisted of a maximum of 25 trials, or 5 min/day. Pretraining consisted of 2 sessions per day, 5 days/week. Preinfarct motor performance and the successful reaching score were recorded on the fourth day before CI. Rats were excluded if the number of trials was fewer than 4 trials at the third week after the start of pretraining, or if preinfarct motor performance was below 20%. The preinfarct motor performance score of most rats improved approximately 40%-60% with training.

Rehabilitative therapy consisted of 2 sessions, 5 days/week, for 4 weeks. Postinfarct motor performance was tested on the fourth day after CI, and weekly thereafter, to monitor functional deficit and recovery. Rats were excluded if the motor performance rate, as a percentage of preinfarct motor performance, was above 25% by the fourth day after CI. Motor performance rate was calculated as follows: {(Preinfarct motor performance – Postinfarct motor performance) ÷ (Postinfarct motor performance)} × 100. To ensure all reaching movements were

analyzed correctly, all test sessions were recorded using a video camera, and the recorded video was analyzed in a blind manner.

Intracortical Microstimulation (ICMS)

Standard ICMS procedures were used to determine the motor map reorganization in the frontal cortex ipsilateral to the ischemic lesion.[22] The rats were anesthetized with ketamine hydrochloride/xylazine (48 mg/kg/4.8 mg/kg, respectively, intraperitoneal), with supplemental continuous ketamine hydrochloride (3.75 mg/kg/h, intravenously, adjustable as needed) to maintain anesthesia. Using bregma as a cranial landmark, a platinum–iridium electrode (1.0-MOhm nominal impedance; World Precision Instruments, Sarasota, FL) was positioned perpendicular to the cortical surface with a .5-mm grid and was lowered to 1.6 mm (approximately cortical layer 5).[23] The inspection grid covered the CFA and the rostral forelimb area (RFA) of the motor cortex (anteroposterior, –3.5~–2.0 to 2.0 mm; mediolateral, 1.5 mm to 5.0 mm from the bregma). Electrical stimulation was delivered as 10 monophasic cathodal pulses (200-μs duration at 333 Hz, 0-80 μA). The absence of detectable movements at the maximum current level of 80 μA was defined as "no response." We recorded the threshold minimum current required for movement. The ICMS procedure was performed by an individual who was blinded to the experimental group.

Cytosine-β-D-Arabinofuranoside (Ara-C) Suppression of Neurogenesis

Ara-C (2%; Sigma-Aldrich, St. Louis, MO) in .9% saline or vehicle only (.9% saline) was continuously infused into the lateral ventricle for 7 days at a rate of 1.0 μL/h, starting on day 4 after CI, with an osmotic minipump (model 2001; Alzet, Palo Alto, CA). The cannula tip was delivered into the ventricle contralateral to the ischemic insult area, 1.6 mm lateral to the midline, .8 mm posterior to the bregma, and 4.0 mm deep from the pial surface.

Briefly, the rats were randomly divided into 2 groups: (1) the vehicle group (n = 4) and (2) the Ara-C group (n = 4). 5-Bromo-2'-deoxyuridine (BrdU) (50 mg/kg) was injected intraperitoneally into the rats daily for 4-10 days after CI. After 7 days of infusion, the rats were perfused for epifluorescent microscopic quantification of BrdU, a marker for neurogenesis, in the SVZ and the peri-infarct cortex (.7 mm anterior from the bregma). To assess the effect of Ara-C treatment on experimental animals, multilabel immunohistochemistry was utilized. Following the completion of ICMS, the rats were perfused as described below for analysis of histological changes and neuronal differentiation. Sections of the peri-infarct cortex, striatum, and corpus callosum (CC) (.7 and .2 mm anterior and .3 mm posterior from the bregma) were used for image analysis. The number of BrdU/Dcx double-labeled cells in the peri-infarct cortex, striatum, and CC were counted per animal (n = 5-6 per group) using epifluorescence microscopy. In addition, the number of BrdU/NeuN, BrdU/Iba-1, BrdU/GFAP, or BrdU/laminin double-labeled cells in the peri-infarct cortex were also counted per animal (n = 4-6 per group) using epifluorescence microscopy. Cell counting was performed using MetaXpress Imaging Analysis V2.0 (Molecular Devices, Tokyo, Japan) by an individual who was blinded to the experimental group.

Time Course of Endogenous Neurogenesis and Inflammatory Response after CI

To analyze the time course of neurogenesis of neural stem cells and microglia after CI in the SVZ and peri-infarct cortical area, BrdU (50 mg/kg) was injected in each rat intraperitoneally on the day before perfusion. Rats were perfused at 2, 4, 7, 14, and 28 days after CI (n = 4 per group). BrdU/nestin or BrdU/Iba-1 double-labeled cells were counted in the SVZ and peri-infarct area (.7 mm posterior from bregma), respectively, using epifluorescence microscopy (BX61; Olympus Corporation, Tokyo, Japan).

Immunohistochemistry and Infarct Volume Measurement

After ICMS, the rats were sacrificed with an overdose of anesthetic and immunohistochemistry was carried out as previously described.[24] Coronal sections (10 μm) were permeabilized for 15 minutes with .1% Triton X-100 and then incubated for 1 hour with blocking buffer containing 2% bovine serum albumin and .1% Triton-X-100 in phosphate-buffered saline at room temperature. The sections were then incubated with mouse anti-BrdU (1:100; Roche Diagnostics, Indianapolis, IN), rabbit anti-nestin (1:1000; R&D Systems Inc., Minneapolis, MN), rabbit anti-NeuN (1:1000; Merck Millipore, Darmstadt, Germany), mouse anti-NeuN (1:1000, Merck Millipore), rabbit anti-Iba-1 (1:1000; Wako Pure Chemical, Osaka, Japan), rabbit anti-GFAP (1:1000; Dako, Carpinteria, CA), rabbit anti-laminin (1:1000, Sigma-Aldrich), goat anti-doublecortin (Sc-8066, 1:500; Santa Cruz Biotechnology, Santa Cruz, CA), or rabbit anti-BDNF (1:200; Proteintech, Rosemont, IL) primary antibodies at 4 °C overnight. The sections were washed 3 times with phosphate-buffered saline and incubated with Alexa Fluor-conjugated secondary antibodies (AF-488, -568; 1:1000; Life Technologies, Carlsbad, CA) at room temperature for 1 hour.

Some sections (10 μm thick, 3.5 mm anterior~3.0 mm posterior from the bregma) were stained for Nissl substance to calculate the infarct volume. Images of these sections were recorded digitally using a scanner, and intact and injured hemisphere areas were measured by a blinded observer using ImageJ software (National Institutes of Health, Bethesda, MD). Infarct volume was calculated as follows: {(Intact hemisphere) – (Injured hemisphere subtracted from the infarct lesion)}/(Intact hemisphere) × 100.[25]

Statistical Analysis

Statistical analyses were performed using SPSS ver. 18 (SPSS Inc., Chicago, IL). The skilled forelimb reaching task was analyzed using 2-way repeated analysis of variance, followed by Tukey's post hoc test. ICMS data were analyzed using 1-way analysis of variance followed by Tukey's post hoc test. Histological data were analyzed using Student's *t*-test. All quantitative data were expressed as mean ± standard deviation. *P* values less than .05 were considered statistically significant.

Results

Effects of Rehabilitative Training on Motor Recovery and Motor Map Reorganization

Three rats died during the experimental procedure, one during photothrombosis and two during ICMS. Four rats were excluded after meeting the exclusion criteria of the skilled forelimb task analysis. Thus, analysis was completed in 39 of 46 rats, which were randomly divided into 4 groups: (1) sham/no-rehab group (n = 10, sham operation without rehabilitative therapy), (2) sham/rehab group (n = 10, sham operation with rehabilitative therapy), (3) CI/no-rehab group (n = 9, CI without rehabilitative therapy), and (4) CI/rehab group (n = 10, CI with rehabilitative therapy). The experimental paradigm is shown in Figure 1, A.

A summary of the skilled forelimb reaching test is illustrated in Figure 1, B. After CI, the rats showed significant motor deficits ($P < .001$, sham/no-rehab versus both CI groups). Motor performance for the rehab group improved from the seventh day and mostly recovered to baseline by the 28th day after CI. On the other hand, functional recovery in the no-rehab group was minimal or nonexistent until the 28th day. Post hoc tests revealed that the CI/rehab group was significantly improved in functional recovery compared to the CI/no-rehab group on both the 21st day (92.16 ± 14.20% versus 35.83 ± 14.68%, $P = .029$) and the 28th day (100.78 ± 15.23% versus 36.88 ± 14.60%, $P = .032$).

As shown in Figure 1, C,D, the CFA was mostly diminished after CI ($P < .001$, sham/no-rehab versus both CI groups). No significant difference related to rehabilitative therapy was observed in any area of the sham groups. In contrast, after CI, the size of the RFA was significantly larger for the rehab group than for the no-rehab group (.69 ± .45 mm^2 versus 1.22 ± .30 mm^2, $P = .003$).

Time Course of Endogenous Neurogenesis and Inflammatory Responses, and Acute Effect of Ara-C Administration after CI

Ara-C treatment inhibits neurogenesis but can also affect microglia. Thus, to optimize Ara-C administration and minimize its effects on microglia, we analyzed the time course of neurogenesis and microglial inflammation immunohistochemically. We performed double immunofluorescence labeling of BrdU/nestin and BrdU/Iba-1 (n = 4 per day per group). The number of BrdU/nestin (neuronal) double-labeled cells in the SVZ increased from the second day, and peaked on the seventh day, after CI. After this point, the number of BrdU/nestin double-labeled cells gradually decreased and returned to its baseline value by the 28th day (Fig 2). The number of BrdU/Iba-1 (microglia) double-labeled cells in the peri-infarct cortex peaked on the second day, and decreased to half the peak value by the seventh day, after CI (Fig 2). Double immunofluorescence labeling of BrdU/nestin and BrdU/Iba-1 was not observed in the peri-infarct cortex and in the SVZ, respectively. These data indicated that significant suppression of neurogenesis with minimal influence on microglial proliferation would be achieved with Ara-C administration beginning on day 4 after CI.

Validation of the Suppression of Endogenous Neurogenesis by Ara-C after CI

To confirm the suppression of neurogenesis, a separate immunohistochemical experiment was performed as described in Figure 3, A. The number of BrdU/nestin double-labeled cells was significantly decreased in the Ara-C group relative to the vehicle group (41 ± 6 versus 179 ± 10/mm^2, $P = .005$). Ara-C treatment decreased endogenous neurogenesis after CI by approximately 80%. However, there were no statistically significant differences in the number of BrdU/Iba-1 double-labeled cells between the vehicle and Ara-C groups (702 ± 89 versus 683 ± 134/mm^2, $P = .85$) (Fig 3, B).

Effects of Ara-C Treatment on Motor Recovery and Motor Map Reorganization Induced by Rehabilitative Therapy after CI

All rats received rehabilitative therapy for 4 weeks after CI. The experimental paradigm is shown in Figure 4, A. Although the general health of the rats was slightly worse for several days after fitting the osmotic minipump, there were no significant differences in body weight or the number of reach trials in rehabilitative training. Any motor abnormalities previously reported in Ara-C administration, such as cooperation motor deficit and trunk imbalance,[26] were not observed. As shown in Figure 4, B, the motor performance for the CI/vehicle group improved from the 14th day, and mostly recovered to baseline by the 28th day after CI, similar to the results in the "rehabilitative therapy" experimental category above. On the other hand, functional recovery in the CI/Ara-C group was suppressed by the 28th day. Post hoc tests revealed that the CI/vehicle group demonstrated significantly improved functional recovery compared to the CI/Ara-C group on the 14th day (60.40 ± 15.08% versus 22.97 ± 7.10%, $P = .003$), the 21st day (85.91 ± 22.69% versus 41.21 ± 13.26%,

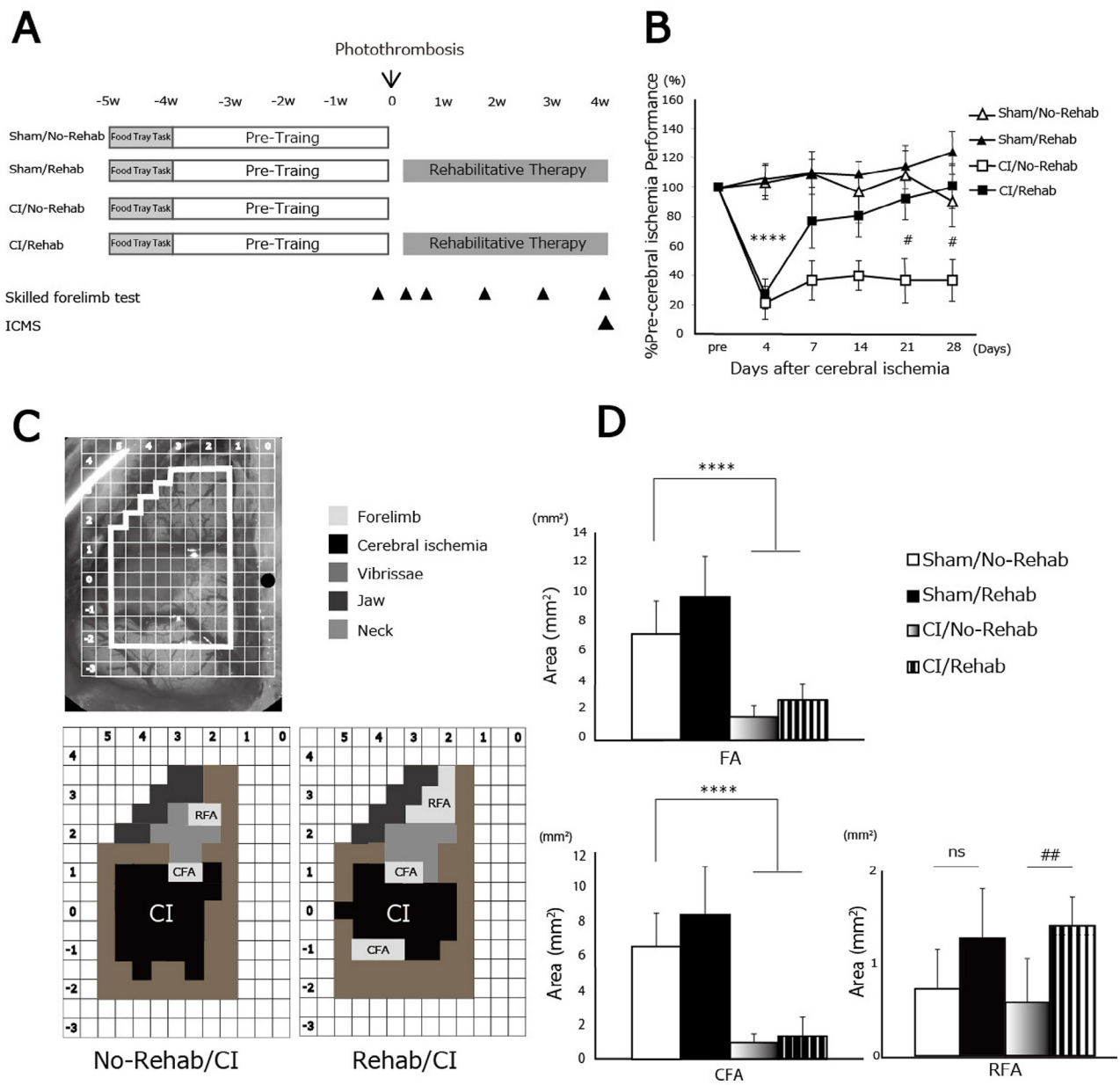

Figure 1. *Effects of rehabilitative therapy on motor recovery and motor map reorganization. (A) Protocol for each group. Rats were divided randomly into 4 groups: sham/no-rehab group (n = 10), sham/rehab group (n = 10), CI/no-rehab group (n = 9), and CI/rehab group (n = 10). All groups were given a food tray task and pretraining for the skilled forelimb task for 5 weeks, after which they underwent photothrombosis or sham surgery. Baseline premotor performance was tested before the lesion. Recovery was monitored for 4 weeks. ICMS was used to study motor map reorganization. (B) Results of the skilled forelimb reaching test. The mean number of successful reaches, scored as a percentage of premotor performance in each of the 4 groups, is shown. (C) Example of a brain motor map. The ICMS mapping area is illustrated on a dorsal view of the brain surface. The area within the bold white line was inspected. The black circle was placed in bregma (x, y = 0, 0). Examples of brain mapping using ICMS in the no-rehab/CI group and the rehab/CI group are shown. The CFA and RFA were present across the neck area. (D). Results of ICMS. The mean areas for the FA, CFA, and RFA movement representations in each of the 4 groups. Data are presented as mean ± standard deviation. Abbreviations: CFA, caudal forelimb area; CI, cerebral ischemia; FA, forelimb area; ICMS, intracortical microstimulation; ns, not significant; Rehab, rehabilitative therapy; RFA, rostral forelimb area. *P < .0001 for the CI/no-rehab and CI/rehab groups compared to the sham/no-rehab group, **P < .05, ***P < .01 for the CI/no-rehab/CI group compared to the CI/rehab group.*

P = .008), and the 28th day (93.29 ± 18.99% versus 41.21 ± 11.23%, P = .001).

A summary of the ICMS results is illustrated in Figure 4, C. No significant difference was observed in any area of the sham group when comparing the vehicle and Ara-C groups. In contrast, when comparing the vehicle and Ara-C groups after CI, the size of the RFA was significantly smaller for the Ara-C group versus the vehicle group (.52 ± .28 mm^2 versus 1.41 ± .65 mm^2, P = .01).

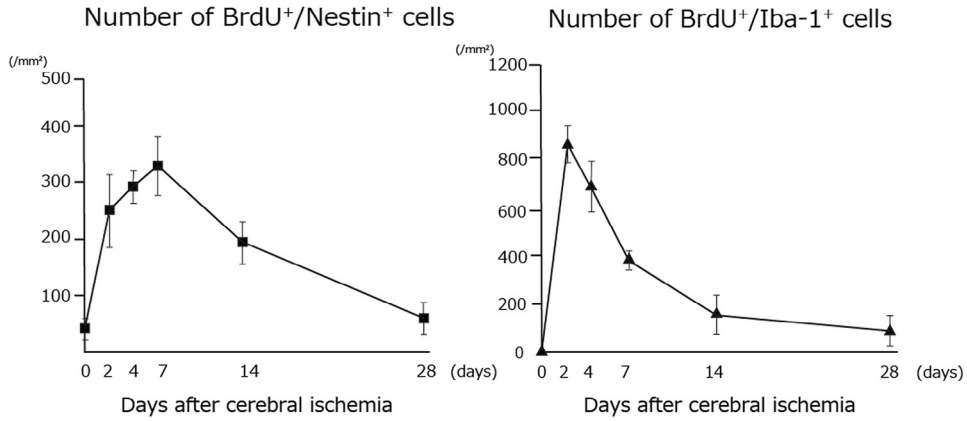

Figure 2. *The time course of endogenous neurogenesis and inflammatory responses after cerebral ischemia. The number of BrdU and nestin double-positive cells in the subventricular zone, or Iba-1 double-positive cells in the peri-infarct cortex. The number of BrdU and nestin double-positive cells peaked on the seventh day, whereas BrdU and Iba-1-positive cells peaked at 2 days after cerebral ischemia. Data are presented as mean ± standard deviation. Abbreviation: BrdU, 5-bromo-2′-deoxyuridine.*

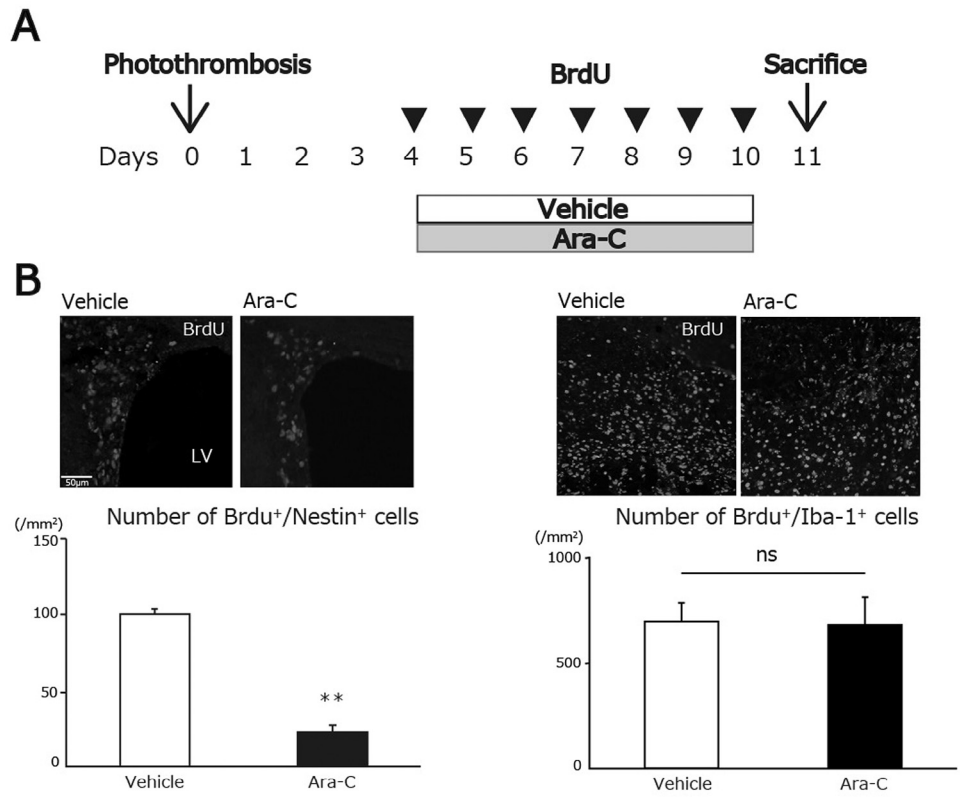

Figure 3. *Effects of Ara-C administration on endogenous neurogenesis and inflammatory responses at 10 days after cerebral ischemia. (A) Protocol for the timing of Ara-C or vehicle treatment. Treatments were continuously infused for 7 days starting on the fourth day after cerebral ischemia, and BrdU was injected daily during this period. (B) Examples and quantification of nestin+, Iba-1+, and BrdU+ cells. The monochrome image shows BrdU+ (white) in the SVZ of the ipsilateral hemisphere to the infarct and peri-infarct lesions. The bar graph shows the number of BrdU+/nestin+ cells and BrdU+/Iba-1+ cells for the vehicle group (n = 5) and the Ara-C group (n = 6) in the SVZ of the ipsilateral hemisphere to the infarct and peri-infarct lesions. The Ara-C group had significantly fewer BrdU+/nestin+ cells compared to the vehicle group (Student's t-test, P = .005). There were no significant differences in the number of BrdU+/Iba-1+ cells in the peri-infarct lesion of the vehicle group compared to the Ara-C group (Student's t-test, P = .85). Data are presented as mean ± standard deviation. Abbreviations: Ara-C, cytosine-β-D-arabinofuranoside; BrdU, 5-bromo-2′-deoxyuridine; ns, no significant difference; SVZ, subventricular zone.*

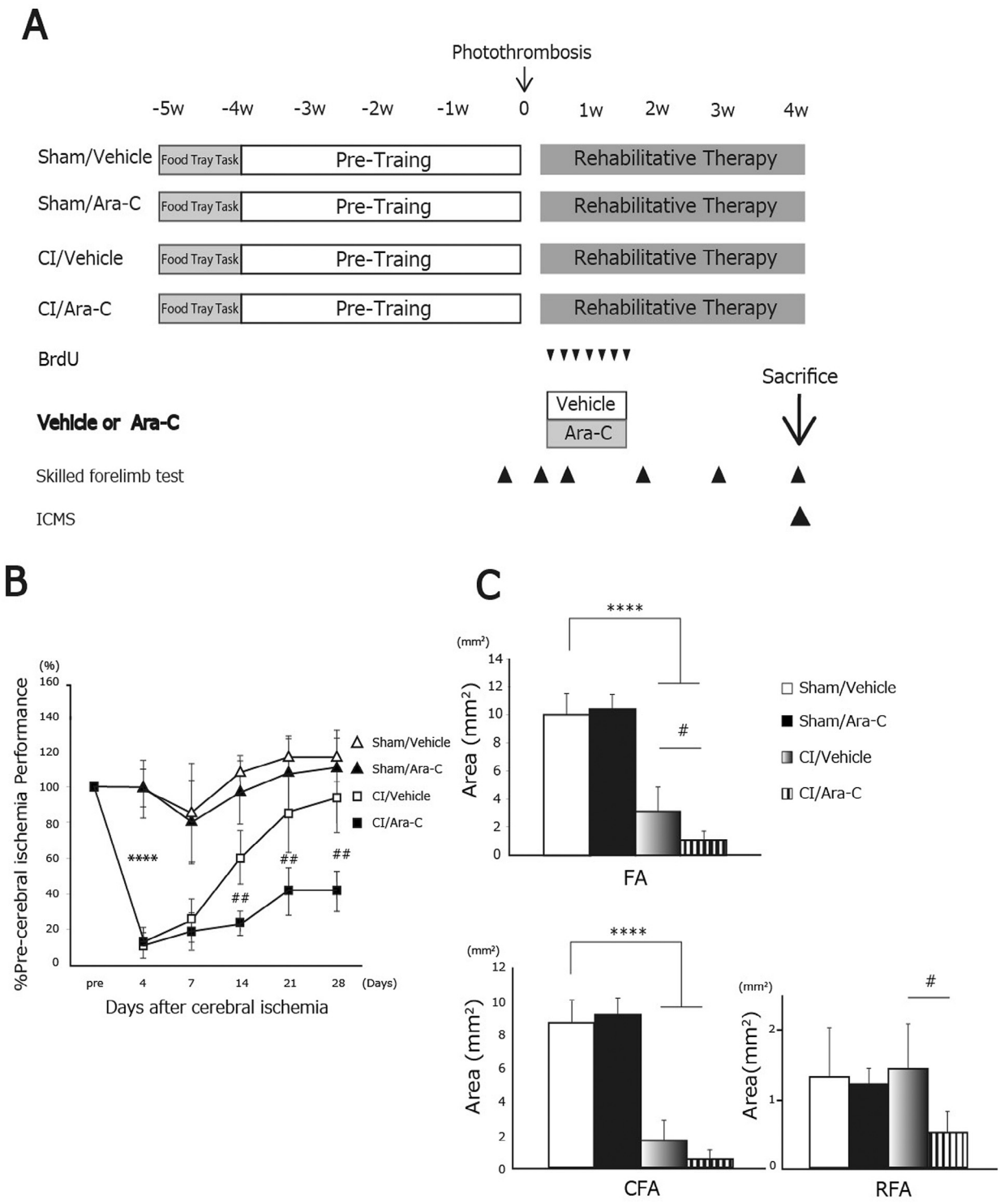

Figure 4. *Effects of inhibition of endogenous neurogenesis on motor recovery and motor map reorganization induced by rehabilitative therapy after cerebral ischemia. (A) Experimental protocol for each group. All rats were randomly divided into 4 groups: vehicle/sham group (n = 9), Ara-C/sham group (n = 9), CI/vehicle group (n = 10), and CI/Ara-C group (n = 10). All groups were provided with a food tray task and pretraining before the skilled forelimb task for 5 weeks. The rats then underwent photothrombosis or sham surgery. Baseline premotor performance was tested before the lesion. All rats received rehabilitative therapy, and recovery was monitored for 4 weeks. Vehicle or Ara-C was continuously infused for 7 days starting on the third day after the lesion, and BrdU was also injected daily during this period. ICMS was used to study motor map reorganization. (B) Results of the skilled forelimb reaching test. The mean number of successful reaches scored as a percentage of premotor performance in each of the 4 groups is shown. (C) Quantification of brain map reorganization. The mean areas for the FA, CFA, and RFA movement representations in each of the 4 groups. Data are presented as mean ± standard deviation. Abbreviations: Ara-C, cytosine-β-D-arabinofuranoside; BrdU, 5-bromo-2′-deoxyuridine; CFA, caudal forelimb area; CI, cerebral ischemia; FA, forelimb area; ICMS, intracortical microstimulation; RFA, rostral forelimb area. *P < .0001 for the sham/CI group compared to the CI/vehicle group and CI/Ara-C group, **P < .05, ***P < .01.*

Effect of Ara-C Treatment on Endogenous Neurogenesis and Non-neural Cells after CI

As shown in Figure 5, A, the mean lesion volume (percent of the contralateral hemisphere) for the vehicle group was 9.11 ± 2.38%, and that for the Ara-C group was 9.78 ± 2.56%. There were no statistically significant differences in the lesion volume between the vehicle and Ara-C groups ($P = .635$). Histological abnormalities related to Ara-C administration, such as neuronal death and glial activation, were absent from the sham groups.

To assess the number of newborn neural precursor cells, mature neurons, and microglia, we performed double immunofluorescence labeling with BrdU/Dcx, NeuN, Iba-1, GFAP, and laminin at 4 weeks after CI. At this time, BrdU-positive cells were not observed in the SVZ but were found in the peri-infarct cortical area, CC, and striatum. Although ICMS revealed significant motor map reorganization in the RFA, neither BrdU/Dcx nor BrdU/NeuN double-labeled cells were observed in the RFA. There were no significant differences in the number of BrdU/Dcx double-labeled cells in both the CC and striatum of the CI/Ara-C group versus the CI/vehicle group (CC; 12.80 ± 2.99 versus 7.75 ± 6.61/mm^2, $P = .22$; striatum; 1.80 ± 1.73 versus .75 ± .71/mm^2, $P = .35$) (Fig 5, B). However, the CI/Ara-C group had significantly fewer BrdU/Dcx double-labeled cells in the peri-infarct cortical area compared to the vehicle/CI group (26.60 ± 6.56 versus 9.0 ± 6.20/mm^2, $P = .009$). Furthermore, the number of BrdU/NeuN double-labeled cells in the CI/Ara-C group was significantly decreased compared to that in the CI/vehicle group (23.41 ± 12.52 versus 8.77 ± 5.27/mm^2, $P = .043$) (Fig 5, C). However, there were no significant differences between the CI/vehicle group and the CI/Ara-C group in the number of BrdU/Iba-l double-labeled cells (190.99 ± 91.70 versus 170.28 ± 23.10/mm^2, $P = .68$) (Fig 6, A), BrdU/GFAP double-labeled cells (144.75 ± 43.68 versus 149.33 ± 47.34/mm^2, $P = .91$) (Fig 6, B), and BrdU/laminin double-labeled cells (50.24 ± 7.74 versus 50.56 ± 4.17/mm^2, $P = .97$) (Fig 6, C) in the peri-infarct cortex. In the sham group, we did not find any cells labeled for BrdU in the peri-infarct cortical area, the CC, or the striatum at 4 weeks after sham operation (data not shown).

Association between Endogenous Neurogenesis and Brain-Derived Neurotrophic Factor (BDNF) after CI

To assess whether newborn neural stem cells release neurotrophic factors such as BDNF, we performed double immunofluorescence labeling with BDNF/nestin and BDNF/NeuN at 7 and 28 days after CI. BDNF/nestin and BDNF/NeuN double-positive cells were observed in the SVZ and peri-infarct area, respectively (Fig 7, A, B).

Discussion

Effects of Rehabilitative Training on Motor Recovery and Motor Map Reorganization

Several studies have demonstrated that rehabilitative therapy after stroke promotes motor recovery and motor map reorganization in human patients,[27] as well as in primate[10] and rodent[22] models. Our experiment showed that skilled forelimb reaching training promoted motor performance and increased the size of the RFA following CFA injury, as has been noted previously.[28] Although motor map reorganization is also known to correlate with the acquisition of motor skills,[29] forelimb training in the sham rats had no significant effects on the motor map. These results suggest that motor map reorganization was associated with rehabilitative training, but not skill acquisition training. The RFA in rodents is generally accepted as homologous to the secondary motor area in humans and primates.[30] In humans and other primates, the secondary motor area, which includes the premotor and supplementary motor areas, reorganizes the representative field and helps to recover lost function.[31,32] Similarly, motor map reorganization in the RFA has also been reported to associate with functional recovery.[28] Therefore, it is very likely that reorganization of the RFA contributed to the functional recovery from motor deficits caused by the damage to the CFA.

Validation of the Suppression of Endogenous Neurogenesis by Ara-C after CI

The antimitotic drug Ara-C has been used to suppress endogenous neurogenesis after CI; however, the treatment affects not only newborn neurons but also non-neural cells such as microglia, astrocytes, and vascular endothelial cells.[33,34] Microglia have a particularly strong influence on motor recovery after CI, as they secrete inflammatory cytokines that can increase postischemic infarct volume,[35] or neurotrophic factors that instead protect the peri-infarct neural tissue.[36] Previous reports also showed that astrocytes and vascular endothelial cells contribute to cerebral remodeling.[37,38] Hence, it is crucial to minimize the effects of Ara-C treatment on non-neural cells to properly evaluate the role of neurogenesis. In the present study, we solved this issue by managing the administration period. We timed our injection protocol such that Ara-C treatment did not show any significant effects on microglial proliferation, either 1 day or 3 weeks after administration, whereas endogenous neurogenesis was strongly suppressed. In addition to the time course, the injection site may also be critical. Because neural stem cells reside and proliferate only in the SVZ, in contrast to microglia, which proliferate mainly in the peri-infarct zone, intraventricular administration of Ara-C should affect neural stem cells much more strongly than microglia. Further histological analysis demonstrated that Ara-C

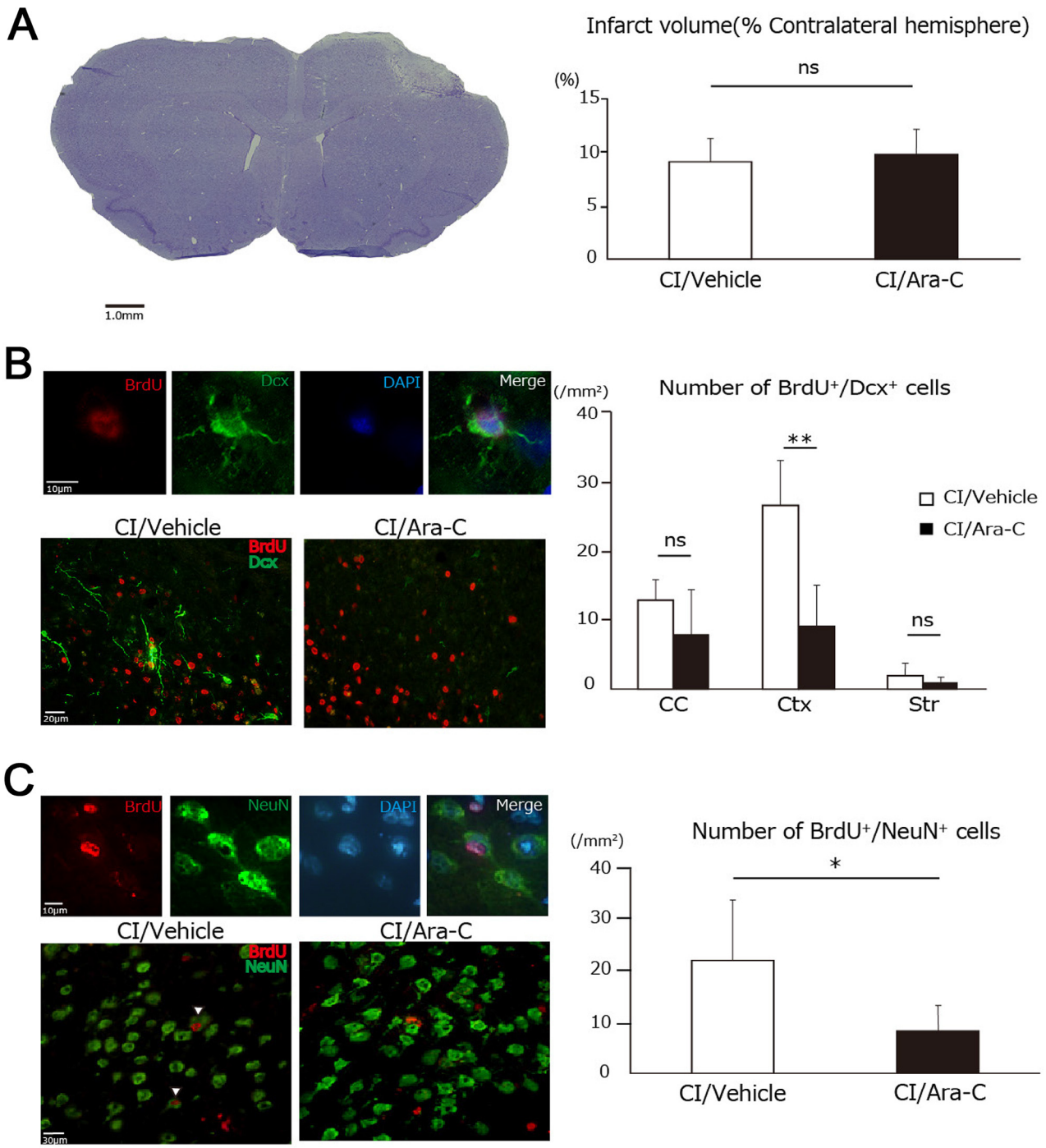

Figure 5. *Effects of Ara-C administration on infarct volume and neural cells at 4 weeks after cerebral ischemia. (A) Histological analysis of the infarct volume. Nissl staining of coronal sections shows the infarct lesion at .7 mm posterior to the bregma. Lesions spread through all layers of the cerebral Ctx but mostly spared the white matter. The bar graph shows the infarct volume as a percentage of the contralateral hemisphere (CI/vehicle group n = 7, CI/Ara-C group n = 6). There were no significant differences between the CI/vehicle group and the CI/Ara-C group (Student's* t*-test,* P = .64*). (B and C) Immunohistochemistry of BrdU and Dcx, NeuN double-positive cells. The photomicrographs show BrdU+ (red), Dcx+, NeuN+ (green), and DAPI+ (blue) in the peri-infarct Ctx. Representative fluorescent images of BrdU and Dcx, NeuN double staining CI/vehicle group and CI/Ara-C group in the Ctx. The bar graph shows the number of BrdU+/Dcx+ and BrdU+/NeuN+ cells for the CI/vehicle group (n = 5) and the CI/Ara-C group (n = 6) in the CC, Ctx, and Str of the hemisphere ipsilateral to the infarct lesion. In the peri-infarct cortical area, the CI/Ara-C group had significantly fewer BrdU+/Dcx+ cells compared to the CI/vehicle group (1-way analysis of variance,* P = .009*). Also, in the peri-infarct cortical area, the CI/Ara-C group had significantly fewer BrdU+/NeuN+ cells compared to the CI/vehicle group (Student's* t*-test,* P = .043*). Data are presented as mean ± standard deviation. Abbreviations: Ara-C, cytosine-β-*D*-arabinofuranoside; BrdU, 5-bromo-2′-deoxyuridine; CC, corpus callosum; CI, cerebral ischemia; Ctx, cortex; DAPI, 4′,6-diamidino-2-phenylindole; ns, no significant difference; Str, striatum. *P < .05 for the CI/Ara-C group compared to the CI/vehicle group,**P < .01 for the CI/Ara-C group compared to the CI/vehicle group. (Color version of figure is available online.)*

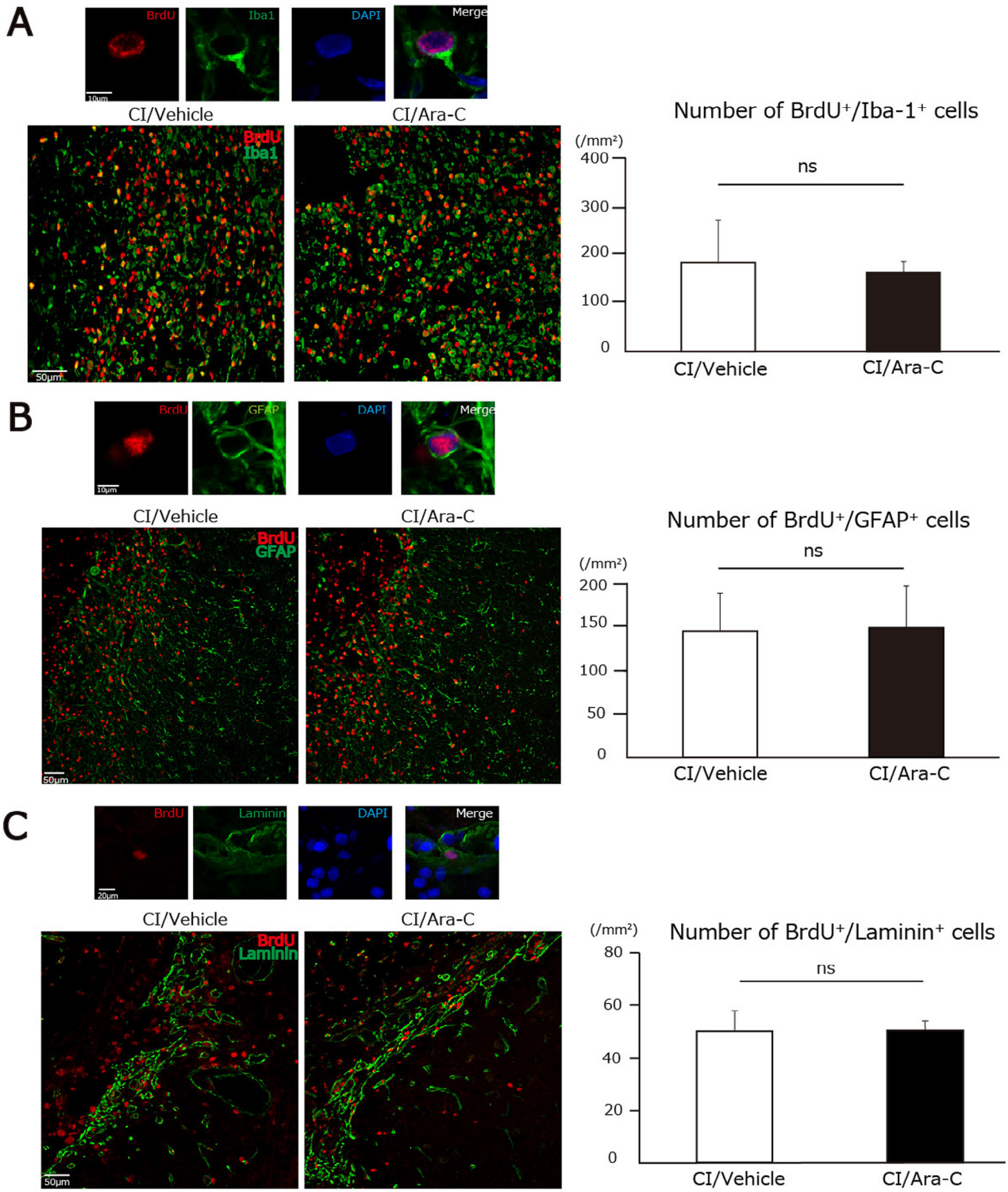

Figure 6. *Effects of Ara-C administration on non-neural cells at 4 weeks after cerebral ischemia. (A-C) Immunohistochemistry of BrdU and Iba-1, GFAP, and laminin double-positive cells. The photomicrographs show BrdU⁺ (red), Iba-1⁺, GFAP⁺, laminin (green), and DAPI⁺ (blue) in the peri-infarct Ctx. Representative fluorescence images of BrdU and Iba-1, GFAP, and laminin double staining CI/vehicle group and CI/Ara-C group in the Ctx. The bar graph shows the number of BrdU⁺/Iba-1⁺, BrdU⁺/GFAP⁺, and BrdU⁺/laminin⁺ cells for the CI/vehicle group (n = 4) and the CI/Ara-C group (n = 5) in the peri-infarct Ctx of the hemisphere ipsilateral to the infarct lesion. There were no statistically significant differences between the CI/vehicle group and the CI/Ara-C group for the number of BrdU⁺/Iba-1⁺ cells (Student's t-test, P = .73), BrdU⁺/GFAP⁺ cells (Student's t-test, P = .91) and BrdU⁺/laminin⁺ cells (Student's t-test, P = .97). Data are presented as mean ± standard deviation. Abbreviations: Ara-C, cytosine-β-D-arabinofuranoside, CI, cerebral ischemia, Ctx, cortex; DAPI, 4′,6-diamidino-2-phenylindole; ns, no significant difference. (Color version of figure is available online.)*

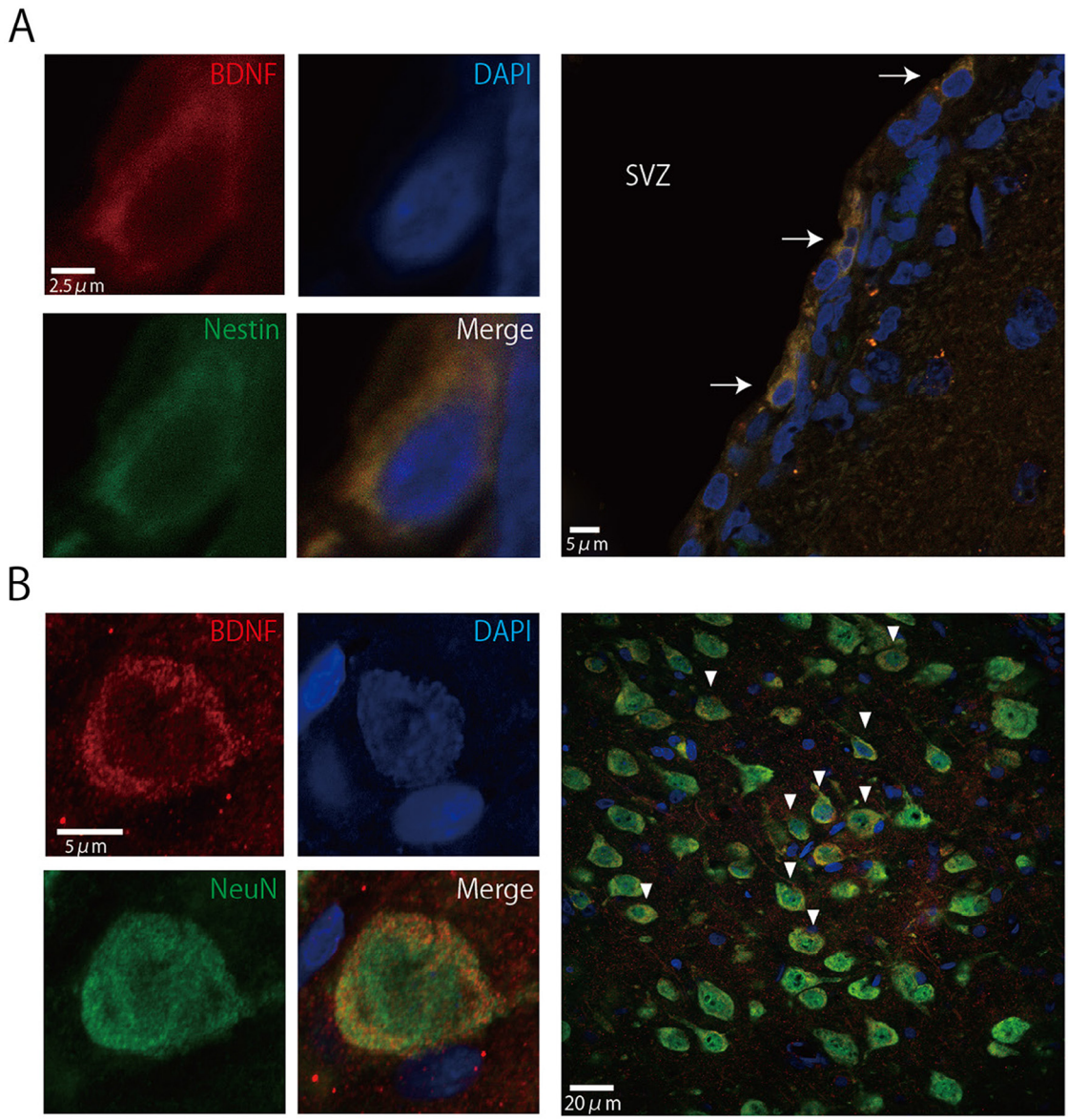

Figure 7. *Histology of BDNF-positive cells after cerebral ischemia. (A) Immunohistochemistry of BDNF and nestin double-positive cells. The photomicrographs show BDNF⁺ (red), nestin⁺ (green), and DAPI⁺ (blue) in the SVZ at 7 days after cerebral ischemia. (B) Immunohistochemistry of BDNF and NeuN double-positive cells. The photomicrographs show BDNF⁺ (red), nestin⁺ (green), and DAPI⁺ (blue) in the peri-infarct cortex at 28 days after cerebral ischemia. Arrows indicate BDNF and nestin double-positive cells. Arrow heads indicate BDNF and NeuN double-positive cells. Abbreviations: BDNF, brain-derived neurotrophic factor; DAPI, 4′,6-diamidino-2-phenylindole; SVZ, subventricular zone (Color version of figure is available online.)*

administration did not show a significant effect either on the proliferation of astrocytes and endothelial cells or on infarct volume. These results suggested that our protocol could inhibit endogenous neurogenesis with a minimal influence on non-neural cells. Furthermore, since Ara-C administration was completed 3 weeks before ICMS measurement, it is not likely that the compound directly affected responsiveness of the neurons in the motor cortex during ICMS. Taken together, although we cannot completely exclude the influence of Ara-C on nonproliferative cells, its effects in our experiment should be primarily mediated by the suppression of endogenous neurogenesis.

Effects of Endogenous Neurogenesis on Motor Recovery and Motor Map Reorganization after CI

Whereas previous reports showed that transgenic ablation of neurogenesis suppressed spontaneous motor recovery,[18,19] the mechanisms underlying the effect were not clear. We showed that inhibition of the endogenous neurogenesis suppressed motor map reorganization as well as motor recovery. These results suggested that endogenous neurogenesis may affect cerebral plasticity after ischemia. In histological analysis at the fourth week after CI, we confirmed that newborn neural stem cells in the

early phase migrated into the damaged peri-infarct area and then differentiated; these neural cells were significantly decreased by Ara-C treatment. However, from previous reports and our data, the number of newborn neurons is supposed to be much smaller than the number of dead neurons to be replaced.[14,39] Arvidsson et al[14] showed that the fraction of dead striatal neurons that had been replaced by the new neurons was as low as about .2%. This fact raises doubts that neurons newly born after CI contribute to functional recovery by directly replacing the dead cells. Additionally, newborn neural cells were not observed in the RFA, where rehabilitative therapy most prominently enhances motor map reorganization. These results indicate that endogenous neurogenesis enhances cerebral plasticity indirectly rather than directly. Several possibilities could explain the mechanisms surrounding the association between endogenous neurogenesis and functional recovery. First, newborn neural stem cells synapse with noninjured neurons and then integrate into the neural network in the CFA.[14,40] Previous reports indicated that the RFA closely interacts both functionally and anatomically with the CFA.[41,42] We suspect that the changes in CFA size related to rehabilitative therapy and the suppression of neurogenesis, though not statistically significant, were indicative of a genuine effect. Therefore, we hypothesize that newborn neurons might modulate neural activity in the RFA by reorganizing the neural network in the CFA. In addition, our experiment suggested that newborn neural stem cells release functionally relevant levels of neurotrophic factors such as BDNF. Those factors play an important role in enhancing cerebral remodeling.[15,19] BDNF is critical in promoting synaptic and axonal plasticity associated with sensorimotor recovery after CI.[43,44] Furthermore, delivery of those factors during the first week after CI is especially important for functional recovery.[45] In the present experiment, Ara-C treatment was performed around the first week after CI and appeared to suppress functional recovery.

In conclusion, our results indicated that rehabilitative therapy promoted motor map reorganization and functional recovery after CI. The restorative effects of rehabilitative therapy were diminished by pharmacological inhibition of endogenous neurogenesis. These results suggest that endogenous neurogenesis may play a critical role in postischemic functional recovery by modulating the cerebral plasticity induced by rehabilitative therapy.

Acknowledgment: We appreciate the central research center section of Kawasaki Medical School.

References

1. Murphy TH, Corbett D. Plasticity during stroke recovery: from synapse to behavior. Nat Rev Neurosci 2009;10:861-872.
2. Dimyan MA, Cohen LG. Neuroplasticity in the context of motor rehabilitation after stroke. Nat Rev Neurol 2011;7:76-85.
3. Brott T, Broderick J, Kothari R, et al. Tissue plasminogen activator for acute ischemic stroke. N Engl J Med 1995;333:1581-1588.
4. Emberson J, Lees KR, Lyden P, et al. Effect of treatment delay, age, and stroke severity on the effects of intravenous thrombolysis with alteplase for acute ischemic stroke: a meta-analysis of individual patient data from randomized trials. Lancet 2014;384:1929-1935.
5. Lee M, Hong KS, Saver JL. Efficacy of intra-arterial fibrinolysis for acute ischemic stroke: meta-analysis of randomized controlled trials. Stroke 2014;41:932-937.
6. Berkhemer OA, Fransen PS, Beumer D, et al. A randomized trial of intra-arterial treatment for acute ischemic stroke. N Engl J Med 2015;372:11-20.
7. Jauch EC, Saver JL, Adams HP Jr, et al. Guidelines for the early management of patients with acute ischemic stroke: a guideline for healthcare professionals from the American Heart Association/American Stroke Association. Stroke 2013;44:870-947.
8. Summers D, Leonard A, Wentworth D, et al. Comprehensive overview of nursing and interdisciplinary care of the acute ischemic stroke patient: a scientific statement from the American Heart Association. Stroke 2009;40:2911-2944.
9. Peter L, Fiona C, Alex P. Motor recovery after stroke: a systematic review. Lancet Neurol 2009;8:741-754.
10. Nudo RJ, Wise BM, SiFuentes F, et al. Neural substrates for the effects of rehabilitative training on motor recovery after ischemic infarct. Science 1996;272:1791-1794.
11. Frost SB, Barbay S, Friel KM, et al. Reorganization of remote cortical regions after ischemic brain injury: a potential substrate for stroke recovery. J Neurophysiol 2003;89:3205-3214.
12. Wittenberg GF. Experience, cortical remapping, and recovery in brain disease. Neurobiol Dis 2010;37:252-258.
13. Gu W, Brannstorm T, Wester P. Cortical neurogenesis in adult rats after reversible photothrombotic stroke. J Cereb Blood Flow Metab 2000;20:1166-1173.
14. Arvidsson A, Collin T, Kirik D, et al. Neuronal replacement from endogenous precursors in the adult brain after stroke. Nat Med 2002;8:963-970.
15. Jin K, Wang X, Xie L, et al. Evidence for stroke-induced neurogenesis in the human brain. Proc Natl Acad Sci USA 2006;103:13198-13202.
16. Zhang L, Hu X, Luo J, et al. Physical exercise improves functional recovery through mitigation of autophagy, attenuation of apoptosis and enhancement of neurogenesis after MCAO in rats. BMC Neurosci 2013;14:46.
17. Zheng HQ, Zhang LY, Luo J, et al. Physical exercise promotes recovery of neurological function after ischemic stroke in rats. Int J Mol Sci 2014;15:10974-10988.
18. Jin K, Wang X, Xie L, et al. Transgenic ablation of doublecortin-expressing cells suppresses adult neurogenesis and worsens stroke outcome in mice. Proc Natl Acad Sci USA 2010;107:7993-7998.
19. Wang X, Mao X, Xie L, et al. Conditional depletion of neurogenesis inhibits long-term recovery after experimental stroke in mice. PLoS ONE 2012;7:e38932.
20. Watson BD, Dietrich WD, Busto R, et al. Induction of reproducible brain infarction by photochemically initiated thrombosis. Ann Neurol 1985;17:497-504.
21. Whishaw IQ, Pellis SM. The structure of skilled forelimb reaching in the rat: a proximally driven movement with

a single distal rotatory component. Behav Brain Res 1990;41:49-59.
22. Kleim JA, Barbay S, Nudo RJ. Functional reorganization of the rat motor cortex following motor skill learning. J Neurophysiol 1998;80:3321-3325.
23. Donoghue JP, Wise SP. The motor cortex of the rat: cytoarchitecture and microstimulation mapping. J Comp Neurol 1982;212:76-88.
24. Okabe N, Nakamura E, Himi N, et al. Delayed administration of the nucleic acid analog 2Cl-C.OXT-A attenuates brain damage and enhances functional recovery after ischemic stroke. Brain Res 2013;1506:115-1131.
25. Swanson RA, Morton MT, Tsao-Wu G, et al. A semiautomated method for measuring brain infarct volume. J Cereb Blood Flow Metab 1990;10:290-293.
26. Patel RS, Rachamalla M, Chary NR, et al. Cytarabine induced cerebellar neurobehavioral performance with cellular and genetic alternations. Toxicology 2012;11:41-52.
27. Gauthier LV, Taub E, Perkins C, et al. Remodeling the brain: plastic structural brain changes produced by different motor therapies. Stroke 2008;15:1520-1525.
28. Conner JM, Chiba AA, Tuszynski MH. The basal forebrain cholinergic system is essential for cortical plasticity and functional recovery following brain injury. Neuron 2005;21:173-179.
29. Plautz EJ, Barbay S, Frost SB, et al. Post-infarct cortical plasticity and behavioral recovery using concurrent cortical stimulation and rehabilitative training: a feasibility study in primates. Neurol Res 2003;25:801-810.
30. Nudo RJ, Frost SB. The evolution of motor cortex and motor systems. In: Kaas JH, Bullock TH, Preuss TM, et al., eds. Evolution of nervous systems. Oxford: Academic Press, 2006:373-395.
31. Miyai I, Suzuki T, Kang J, et al. Middle cerebral artery stroke that includes the premotor cortex reduces mobility outcome. Stroke 1999;7:1380-1383.
32. Liu Y, Rouiller EM. Mechanisms of recovery of dexterity following unilateral lesion of the sensorimotor cortex in adult monkeys. Exp Brain Res 1999;128:149-159.
33. Audet JN, Gowing G, Paradis R, et al. Ablation of proliferating cells in the CNS exacerbates motor neuron disease caused by mutant superoxide dismutase. PLoS ONE 2012;4:e34932.
34. Zhang Y, Chopp M, Mahmood A, et al. Impact of inhibition of erythropoietin treatment-mediated neurogenesis in the dentate gyrus of the hippocampus on restoration of spatial learning after traumatic brain injury. Exp Neurol 2012;235:336-344.
35. Barone FC, Arvin B, White RF, et al. Tumor necrosis factor-alpha. A mediator of focal ischemic brain injury. Stroke 1997;28:1233-1244.
36. Macrez R, Ali C, Toutirais O, et al. Stroke and the immune system: from pathophysiology to new therapeutic strategies. Lancet Neurol 2011;10:471-480.
37. Pannasch U, Vargova L, Reingruber J, et al. Astroglial networks scale synaptic activity and plasticity. Proc Natl Acad Sci USA 2011;108:8467-8472.
38. Hermann DM, Chopp M. Promoting brain remodelling and plasticity for stroke recovery: therapeutic promise and potential pitfalls of clinical translation. Lancet Neurol 2012;11:369-380.
39. Bacigaluppi M, Pluchino S, Martino G, et al. Neural stem/precursor cells for the treatment of ischemic stroke. J Neurol Sci 2008;265:73-77.
40. Lai B, Mao XO, Xie L, et al. Electrophysiological neurodifferentiation of subventricular zone-derived precursor cells following stroke. Neurosci Lett 2008;442:305-308.
41. Deffeyes JE, Touvykine B, Quessy S, et al. Interactions between rostral and caudal cortical motor areas in the rat. J Neurophysiol 2015;113:3893-3904.
42. Jacobs KM, Donoghue JP. Reshaping the cortical motor map by unmasking latent intracortical connections. Science 1991;251:944-947.
43. Schäbitz WR, Berger C, Kollmar R, et al. Effect of brain-derived neurotrophic factor treatment and forced arm use on functional motor recovery after small cortical ischemia. Stroke 2004;35:992-997.
44. Schäbitz WR, Steigleder T, Cooper-Kuhn CM, et al. Intravenous brain-derived neurotrophic factor enhances poststroke sensorimotor recovery and stimulates neurogenesis. Stroke 2007;38:2165-2172.
45. Kokaia Z, Thored P, Arvidsson A, et al. Regulation of stroke-induced neurogenesis in adult brain—recent scientific progress. Cereb Cortex 2006;16:i162-i167.