# Synergic Effects of Rehabilitation and Intravenous Infusion of Mesenchymal Stem Cells After Stroke in Rats

Yuichi Sasaki, Masanori Sasaki, Yuko Kataoka-Sasaki, Masahito Nakazaki, Hiroshi Nagahama, Junpei Suzuki, Daiki Tateyama, Shinichi Oka, Takahiro Namioka, Ai Namioka, Rie Onodera, Takeshi Mikami, Masahiko Wanibuchi, Masafumi Kakizawa, Sumio Ishiai, Jeffery D. Kocsis, Osamu Honmou

**Background.** Intravenous infusion of mesenchymal stem cells (MSCs) derived from adult bone marrow improves behavioral function in rat stroke models. Rehabilitation therapy through physical exercise also provides therapeutic efficacy for cerebral ischemia.

**Objective.** The purpose of this study was to investigate whether synergic effects of daily rehabilitation and intravenous infusion of MSCs has therapeutic effects after stroke in rats.

**Design.** This was an experimental study.

**Methods.** A permanent middle cerebral artery occlusion (MCAO) was induced by intraluminal vascular occlusion with a microfilament. Four experimental groups were studied: group 1 (vehicle only, n=10), group 2 (vehicle + exercise, n=10), group 3 (MSCs only, n=10), and group 4 (MSCs + exercise, n=10). Rat MSCs were intravenously infused at 6 hours after MCAO, and the rats received daily rehabilitation with treadmill running exercise for 20 minutes. Lesion size was assessed at 1, 14, and 35 days using magnetic resonance imaging. Functional outcome was assessed using the Limb Placement Test.

**Results.** Both combined therapy and MSC infusion reduced lesion volume, induced synaptogenesis, and elicited functional improvement compared with the groups without MSC infusion, but the effect was greater in the combined therapy group.

**Limitations.** A limitation of this study is that the results were limited to an animal model and cannot be generalized to humans.

**Conclusions.** The data indicate that the combined therapy of daily rehabilitation and intravenous infusion of MSCs improved functional outcome in a rat MCAO model.

Y. Sasaki, PT, PhD, Department of Neural Regenerative Medicine, Research Institute for Frontier Medicine, Sapporo Medical University School of Medicine, Sapporo, Hokkaido, Japan, and Department of Rehabilitation, Sapporo Medical University School of Medicine.

M. Sasaki, MD, PhD, Department of Neural Regenerative Medicine, Research Institute for Frontier Medicine, Sapporo Medical University School of Medicine, Sapporo, Hokkaido, 060-8556, Japan; Department of Neurology, Yale University School of Medicine, New Haven, Connecticut; and Center for Neuroscience and Regeneration Research, VA Connecticut Healthcare System, West Haven, Connecticut. Address all correspondence to Dr Sasaki at: msasaki@sapmed.ac.jp.

Y. Kataoka-Sasaki, MD, PhD, Department of Neural Regenerative Medicine, Research Institute for Frontier Medicine, Sapporo Medical University School of Medicine.

M. Nakazaki, MD, Department of Neural Regenerative Medicine, Research Institute for Frontier Medicine, Sapporo Medical University School of Medicine.

H. Nagahama, RT, Department of Neural Regenerative Medicine, Research Institute for Frontier Medicine, Sapporo Medical University School of Medicine.

J. Suzuki, PhD, Department of Neural Regenerative Medicine, Research Institute for Frontier Medicine, Sapporo Medical University School of Medicine.

D. Tateyama, BS, Department of Neural Regenerative Medicine, Research Institute for Frontier Medicine, Sapporo Medical University School of Medicine.

*Author information continues on next page.*

S. Oka, MD, PhD, Department of Neural Regenerative Medicine, Research Institute for Frontier Medicine, Sapporo Medical University School of Medicine.

T. Namioka, MD, Department of Neural Regenerative Medicine, Research Institute for Frontier Medicine, Sapporo Medical University School of Medicine.

A. Namioka, MD, Department of Neural Regenerative Medicine, Research Institute for Frontier Medicine, Sapporo Medical University School of Medicine.

R. Onodera, PhD, Department of Neural Regenerative Medicine, Research Institute for Frontier Medicine, Sapporo Medical University School of Medicine.

T. Mikami, MD, PhD, Department of Neurosurgery, Sapporo Medical University School of Medicine.

M. Wanibuchi, MD, PhD, Department of Neurosurgery, Sapporo Medical University School of Medicine.

M. Kakizawa, PT, Department of Rehabilitation, Sapporo Medical University School of Medicine.

S. Ishiai, MD, PhD, Department of Rehabilitation, Sapporo Medical University School of Medicine.

J.D. Kocsis, PhD, Department of Neurology, Yale University School of Medicine, and Center for Neuroscience and Regeneration Research, VA Connecticut Healthcare System.

O. Honmou, MD, PhD, Department of Neural Regenerative Medicine, Research Institute for Frontier Medicine, Sapporo Medical University School of Medicine; Department of Neurology, Yale University School of Medicine; and Center for Neuroscience and Regeneration Research, VA Connecticut Healthcare System.

[Sasaki Y, Sasaki M, Kataoka-Sasaki Y, et al. Synergic effects of rehabilitation and intravenous infusion of mesenchymal stem cells after stroke in rats. *Phys Ther.* 2016; 96:1791–1798.]



Published Ahead of Print:
May 12, 2016
Accepted: May 3, 2016
Submitted: September 10, 2015

Intravenous infusion of mesenchymal stem cells (MSCs) after cerebral stroke can reduce infarction size and improve functional outcome in experimental stroke models.[1–5] Suggested therapeutic mechanisms of MSCs in various models of central nervous system diseases include secretion of neurotrophic factors, which can provide for neuroprotection; reduction in inflammation; neovascularization; and synaptogenesis.[6–8] Clinical studies using intravenous infusion of MSCs in patients with stroke have been carried out or are ongoing.[8,9]

Rehabilitation therapy through physical exercise has demonstrated therapeutic efficacy in experimental animal models, including animals with cerebral ischemia[10–12] and spinal cord injury.[13] Moreover, rehabilitation therapy is efficacious in patients with stroke.[14] Among several approaches for stroke rehabilitation, it has been reported that treadmill exercise could be powerful tool for gait training.[15,16] Previous studies in animal models with cerebral ischemia suggest that the improved functional outcome following rehabilitation is associated with enhanced levels of endogenous neurotrophic factors.[10,17]

In the present study, we investigated the synergistic effects on behavioral function of a combination strategy of rehabilitation (daily treadmill exercise) and intravenous infusion of MSCs 6 hours after induction of unilateral permanent cerebral ischemia in rats.

# Method

## Preparation of MSCs From Rat Bone Marrow

The use of animals in this study was approved by the Animal Care and Use Committee of Sapporo Medical University (#13-125), and all procedures were carried out in accordance with institutional guidelines. The method of MSC culture was based on our previous studies.[18,19] Briefly, bone marrow was obtained from femoral bones in adult Sprague-Dawley rats or green fluorescent protein (GFP)–expressing rats (W-Tg-[CAG-GFP]184Ys); diluted to 25 mL with Dulbecco's modified Eagle's medium (DMEM) (SIGMA, St Louis, Missouri) supplemented with 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific Inc, Waltham, Massachusetts), 2 mM l-glutamine (SIGMA), 100 U/mL penicillin, and 0.1 mg/mL streptomycin (Thermo Fisher Scientific Inc); and incubated for 3 days (5% $CO_2$, 37°C). When cultures almost reached confluence, the adherent cells were detached with trypsin-ethylenediaminetetraacetic acid (EDTA) solution (SIGMA) and subcultured at $1 \times 10^4$ cells/mL. In the present study, we used MSCs after 3 passages.

## Cerebral Ischemic Model

The rat middle cerebral artery occlusion (MCAO) model was used as a stroke model. We induced permanent MCAO by using a previously described method of intraluminal vascular occlusion.[4,20,21] Adult female Sprague-Dawley rats (200–250 g) were anesthetized with an intraperitoneal injection of ketamine (75 mg/kg) and xylazine (10 mg/kg). A length of 20.0- to 22.0-mm 3-0 surgical Monosof suture (Medtronic, Minneapolis, Minnesota) with the tip rounded by heating near a flame was advanced from the external carotid artery into the lumen of the internal carotid artery until it blocked the origin of the MCA.

## Rehabilitation

Treadmill running exercise was performed for 20 minutes every day after MCAO. Exercise started 1 day after MCAO at a speed of 3 m/min with a slope of 0 degrees for 1 week, and speed was increased by 3 m/min every week until histological evaluation.

## MSC Infusion Procedures

The MSC infusion protocols consisted of 4 groups. After the initial diffusion-weighted imaging at 6 hours after MCAO, the rats were randomized into 4 experimental groups. In group 1 (vehicle only, n=10), rats were injected intravenously with vehicle alone (fresh DMEM) through the femoral vein (without donor cell administration) 6 hours after MCAO (just after the initial diffusion-weighted imaging). In group 2 (vehicle + exercise, n=10), rats were infused with DMEM and received daily exercise (see Rehabilitation section). In group 3 (MSCs only, n=10), rats were injected intravenously with MSCs ($1.0 \times 10^6$ cells each) in 1 mL total fluid volume (fresh DMEM) 6

hours after MCAO. In group 4 (MSCs + exercise, n=10), rats were infused with MSCs and received exercise (see Rehabilitation section). All rats, including sham control rats and age-matched intact animals (n=10), were injected daily with cyclosporine A (10 mg/kg, intraperitoneally).[19,22–27]

## Magnetic Resonance Imaging Studies and Measurement of Infarct Volume and Corpus Callosum Thickness

Rats were anesthetized with ketamine (75 mg/kg) and xylazine (10 mg/kg, intraperitoneally). Each rat was placed in an animal holder/magnetic resonance imaging (MRI) probe apparatus and positioned inside the magnet. The animal's head was held in place inside the imaging coil. All MRI measurements were performed using a 7-tesla, 18-cm-bore superconducting magnet (Oxford Instruments, Oxfordshire, United Kingdom) interfaced to a Unity Inova console (Oxford Instruments and Varian Inc, Palo Alto, California) (n=10 per group) described previously.[5,28]

Briefly, diffusion-weighted images were obtained from a 1.0-mm-thick coronal section with a 0.5-mm gap using a 30-mm × 30-mm field of view (repetition time=3,000 milliseconds, echo time=37 milliseconds, b value=1,000 s/mm$^2$) and reconstructed using a 128 × 128 image matrix. The T2-weighted images were obtained from a 1.0-mm-thick coronal section with a 0.5-mm gap using a 30-mm × 30-mm field of view (repetition time=3,000 milliseconds, echo time=30 milliseconds) and reconstructed using a 256 × 256 image matrix. Accurate positioning of the brain was performed to center the image slice 5 mm posterior to the rhinal fissure with the head of the rat held in a flat skull position. Diffusion-weighted images were obtained 6 hours after MCAO, and T2-weighted images were obtained 1, 14, and 35 days after MCAO.

The ischemic lesion area was calculated from T2-weighted images using imaging software (Scion Image, Version Beta 4.0.2, Scion Corporation, Frederick, Maryland) based on the previously described method.[29] Briefly, after optimal adjustment of contrast, the edge of the lesions, where the signal intensity was 1.25 times higher than the counterpart in the contralateral brain lesion, was traced manually on each of the 9 coronal slices, which completely covered the middle cerebral artery territory in all animals. The areas of hyperintensity were then summed and multiplied by the slice thickness plus interslice gap to calculate lesion volumes. It should be noted that the high-intensity area of T2-weighted images at 6 hours is not clear enough to measure stroke volume. However, the high-intensity area of diffusion-weighted images at 6 hours can be used to evaluate the initial stroke volume after MCAO induction. We used criteria for standardizing the initial stroke volume (300 mm$^3$ ± 60 mm$^3$) and excluded deviated animals (the inclusion rate was 25%–30%). Then, the rats were randomized into the 4 experimental groups. Corpus callosum (CC) thickness was measured with T2-weighted images using NIH Image J Analysis Software (version 1.39, National Institutes of Health, Bethesda, Maryland) at the midline on bregma −0.26 mm level slices (modified from Paxino and Watson[30]; Fig. 3A) (n=10 per group).

## Limb Placement Test

In the Limb Placement Test (LPT), the rats' 4 limbs were evaluated using the top and edges of a countertop.[22,31] Each test was scored as follows: 0=no placement, 1=incomplete or delayed >2 seconds placement, and 2=immediate and correct placement. For each body side, the maximum score from the tests used was 16. The forepaws were graded in all 6 tests; in tests 4 and 6, the hind limbs also were tested. During tests 1 through 4, the rat was held in a soft grip by the examiner.

In test 1, limb placement was tested by slowly lowering the rat toward a table. At 10 cm above the table, normal rats stretch and place both forepaws on the table. For test 2, with the rats' forelimbs touching the table edge, the head of the rat was moved 45 degrees upward while the chin was supported to prevent the nose and the vibrissae from touching the table. A rat with a focal brain lesion may lose contact with the table with the paw contralateral to the injured hemisphere. In test 3, forelimb placement of the rat when facing a table edge was observed. A normal rat places both forepaws on the tabletop. Test 4 recorded forelimb and hind-limb placement when the lateral side of the rat's body was moved toward the table edge. For test 5, the rat was placed on the table and gently pushed from behind toward the table edge. A normal rat will grip on the edge, but an injured rat may drop the forelimb contralateral to the injured hemisphere. Test 6 was the same as test 5, but the rat was pushed laterally toward the table edge. Intact animals were used for control.

## Immunohistochemistry

Rats were perfused transcardially with cold phosphate-buffered saline (PBS) followed by 4% paraformaldehyde under deep anesthesia. Whole brains were dissected out, post-fixed in 4% paraformaldehyde overnight, and cryoprotected in 30% sucrose/phosphate-buffered saline at 4°C. Samples were stored at −80°C until use. Coronal sections were cut to 50-$\mu$m thickness using a cryostat (Sakura Seiki Co, Tokyo, Japan). One section per animal was selected according to the rat stereotaxic atlas (bregma −0.4 mm; modified from Paxino and Watson[30]; Fig. 2A), washed in PBS-0.1% Tween 20 (PBS-T) 3 times, blocked in 5% normal donkey serum/0.3 % Triton X-100 in PBS at room temperature for 30 minutes, and incubated in primary antibodies diluted in 5% normal donkey serum/0.3% Triton X-100 /PBS at 4°C overnight. We used rabbit anti-synaptophysin antibody (1:500; Novus/NB110-57606) as a presynaptic marker, which is a presynaptic vesicle protein and indicator of presynaptic plasticity and synaptogenesis,[32] and chicken anti-GFP antibody (1:1,000; Abcam, ab13970) for detection of GFP. After washing in PBS-T 4 times, sections were incubated in secondary antibodies, which were Cy3-conjugated donkey anti-rabbit immunoglobulin G (1:1,000; Jackson/711-165-152) for synaptophysin and AF 488-conjugated goat anti-chicken immunoglobulin Y (1:1,000; Abcam, 150173) for GFP, counter-stained with 4',6-diamidino-2-phenylindole (DAPI), and coverslipped with VECTASHIELD

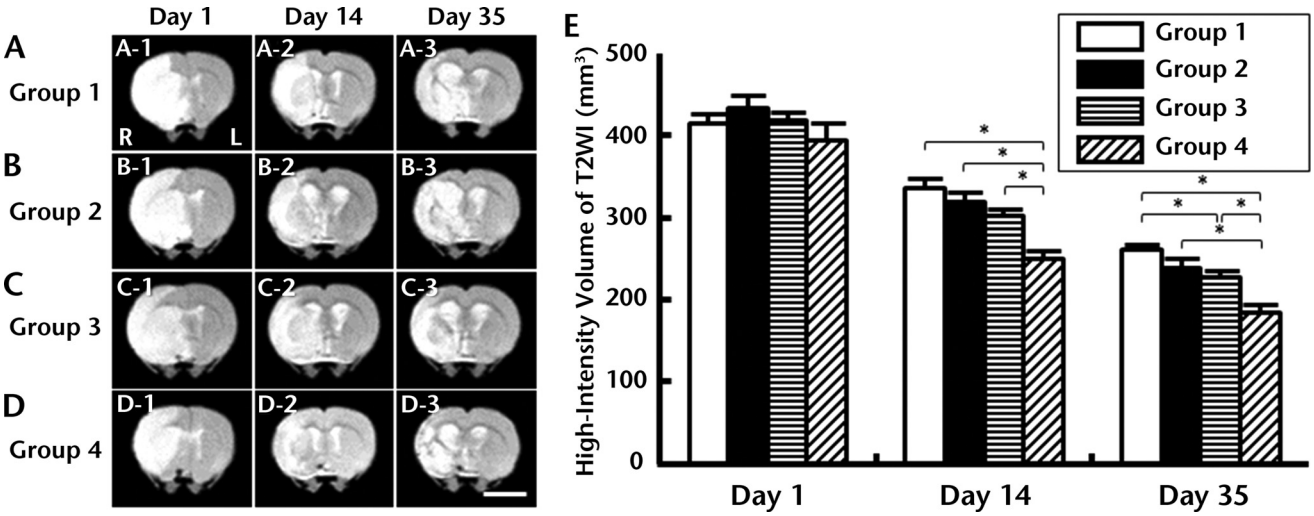


**Figure 1.**
Evaluation of the ischemic lesion volume with magnetic resonance imaging T2-weighted images (T2WI). Rat mesenchymal stem cells (MSCs) or vehicle (fresh Dulbecco's modified Eagle's medium) were intravenously infused 6 hours after middle cerebral artery occlusion (MCAO). The T2WI were obtained at days 1, 14, and 35 from the 4 experimental groups after MCAO induction: (A) group 1 (vehicle only), (B) group 2 (vehicle + exercise), (C) group 3 (MSCs only), and (D) group 4 (MSCs + exercise). Scale bar=5 mm. (E) A summary of lesion volume evaluated with T2WI were obtained 1, 14, and 35 days after MCAO in the 4 experimental groups. R=right, L=left. * $P<.01$.

(Vector Laboratories, Burlingame, California).

### Quantitative Analysis of Synaptic Puncta

The sections were examined using a confocal microscopy with Ex/Em (405; 561: LSM780 ELYRA S.1 system). For quantitative analysis of synaptophysin, confocal images were collected from layer III/IV in the motor cortex in the infarcted hemisphere lesion according to the rat stereotaxic atlas (bregma 0.4 mm: modified from Paxino and Watson[30] Fig. 2A). The regions of interests (ROIs; 2,048 × 2,048 pixels) were placed to quantify the expression of synaptophysin. The coordinate relative to the bregma for the center of the ROI was on the section: −0.40 mm caudal, 3 mm lateral, 0.5 mm depth.[23] Gain thresholds and amplitude offsets were kept constant between imaging of the motor cortex in infarcted hemisphere and homotopic contralateral areas for each section. Averaged synaptic puncta per field were counted using the NIH Image J Analysis Software (version 1.39) in each scanned field (3 fields per animal) from group 1 (n=6 rats), group 2 (n=6 rats), group 3 (n=6 rats), and group 4 (n=6 rats) after MCAO and from intact animals (n=5 rats).

### Data Analysis

All statistical analysis was performed using SPSS 18 (SPSS Inc, Chicago, Illinois). Differences among groups were assessed by analysis of variance with the Scheffé post hoc test. The behavioral data were further tested for a correlation to the density of the synaptic puncta and the thickness of the CC (Pearson correlation).

## Results

### Characterization of Ischemic Lesion Size by MRI Analysis

The ischemic lesion size was estimated for the 4 experimental groups using in vivo MRI (see Method section). The T2-weighted images were obtained from the 4 groups at day 1, day 14, and day 35 after MCAO induction (Fig. 1A–D). These coronal forebrain sections were obtained at the level of caudate-putamen complex. Diffusion-weighted images also were obtained at 6 hours and confirmed no difference of the initial stroke volume among the groups (group 1=293.5±34.7 $mm^3$, group 2=312±28.5 $mm^3$, group 3=305.1±30.1.7 $mm^3$, and group 4=294.8±40.6 $mm^3$; n=10 per group; $P=.619$).

Lesion volume ($mm^3$) was determined by analysis of T2-weighted image high-intensity areas on serial images collected through the cerebrum (see Method section). In all groups, T2-weighted image–estimated lesion volume gradually decreased over the time course of 35 days post-MCAO (Fig. 1E). Group 2 did not show lesion volume reduction at day 14 or day 35 compared with group 1, but group 3 displayed greater volume reduction compared with the control rats at day 35 ($P<.01$) (Fig. 1E). The reduction in lesion volume was greatest for group 4 compared with the other 3 groups at day 14 and day 35 ($P<.01$) (Fig. 1E).

Collectively, these results indicate that the combination strategy of rehabilitation and intravenous infusion of MSCs enhanced reduction of ischemic lesion volume.

### Detection of GFP-MSCs In Vivo

Histological analysis at 3 days post-GFP-MSCs (green) infusion indicated that the infused cells survived and distributed to the infarcted hemisphere (Fig. 2A–B). There were few GFP-MSCs in the contralateral hemisphere. To determine whether autofluorescence of the MSCs was present at the wavelengths used to

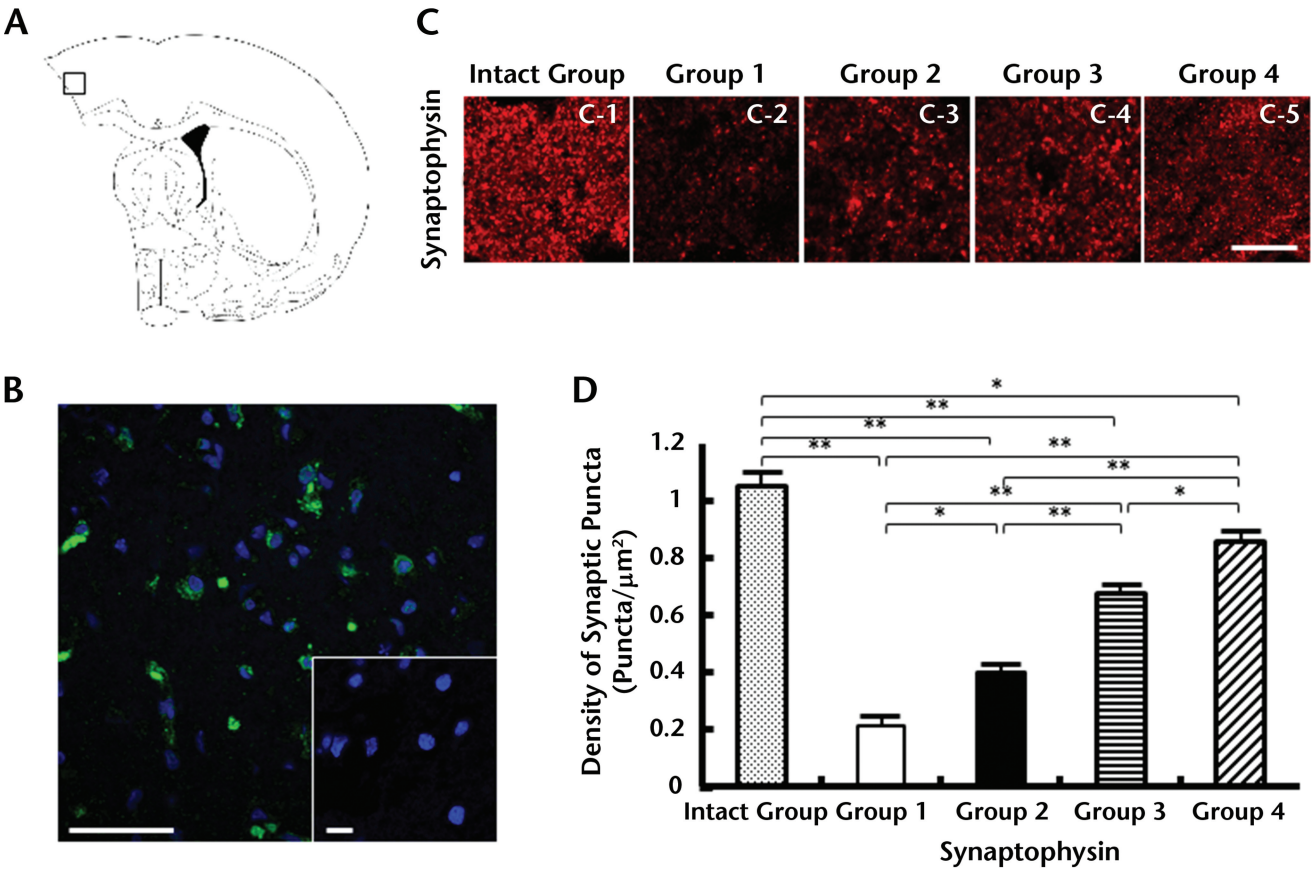


**Figure 2.**
Synaptogenesis in the motor cortex in the infarcted hemisphere at layer III/IV was analyzed with a confocal microscopy. (A) Images were obtained from a boxed area in the motor cortex in infarcted hemisphere. (B) Infused green flourescent protein-mesenchymal stem cells GFP-MSCs (green) were present in the motor cortex in the infarcted hemisphere counterstained with DAPI (blue) 3 days after middle cerebral artery occlusion (n=3). Green flourescent protein (green) was observed in the cytoplasm, and DAPI is stained nucleus. No green cells were observed in these lesions after infusion of MSCs derived from wild-type Sprague-Dawley rats (inset). (C) Representative images of immunohisotochemical study for synaptophysin from intact animals (C-1), group 1 (vehicle only) (C-2), group 2 (vehicle + exercise) (C-3), group 3 (MSCs only) (C-4), and group 4 (MSCs + exercise) (C-5). (D) Quantitative analysis of density of synaptic puncta in the intact and 4 experimental groups. * $P<.05$. ** $P<.01$. Scale bar=10 $\mu$m.

study GFP fluorescence, we examined these sections of animals infused MSCs derived from wild-type Sprague-Dawley rats. No green cells were observed in these lesions (Fig. 2B inset).

## Quantification of Synaptic Density

To determine whether there are synaptic changes following 4 experimental conditions, synaptic density in the motor cortex in the infarcted hemisphere was examined at 6 weeks after stroke induction. Synaptophysin was used to quantify synaptic density (Fig. 2C). In groups 3 and 4, the density of synaptic puncta in the motor cortex in the infarcted hemisphere was higher than in the other non–MSC-infused groups (Fig. 2D). Synaptic density as studied in a defined area of interest (Fig. 2A) was reduced for the 4 experimental groups compared with the intact cortex (Fig. 2D). However, in groups 2, 3, and 4, each group had a greater synaptic density compared with group 1, as summarized in Fig. 2D. The group 4 showed the greatest increase in synaptic density (Fig. 2D). These data suggest that although rehabilitation and MSC infusion each enhance synaptogenesis, the combined therapy has the greatest effect.

## Corpus Callosum Thickness

Quantification of the thickness of the CC was evaluated by T2-weighted images (see Method section) and revealed that the CC was reduced in group 1 (group 1: 135.09±16.14 $\mu$m; intact animals: 329.67±16.81 $\mu$m; $P<.01$; Fig. 3). Corpus callosum thickness was greater in the other 3 interventional groups. Although CC thickness was greater in the group 2 compared with group 1 (group 2: 202.11±0.7 $\mu$m, $P<.01$; Fig. 3B), both group 3 (272.34±44.72 $\mu$m) and group 4 (313.81±28.65 $\mu$m) had greater CC thickness than the group 2 ($P<.01$; Fig. 3B). These data indicate that MSC delivery alone or in combination with rehabilitation preserves CC thickness.

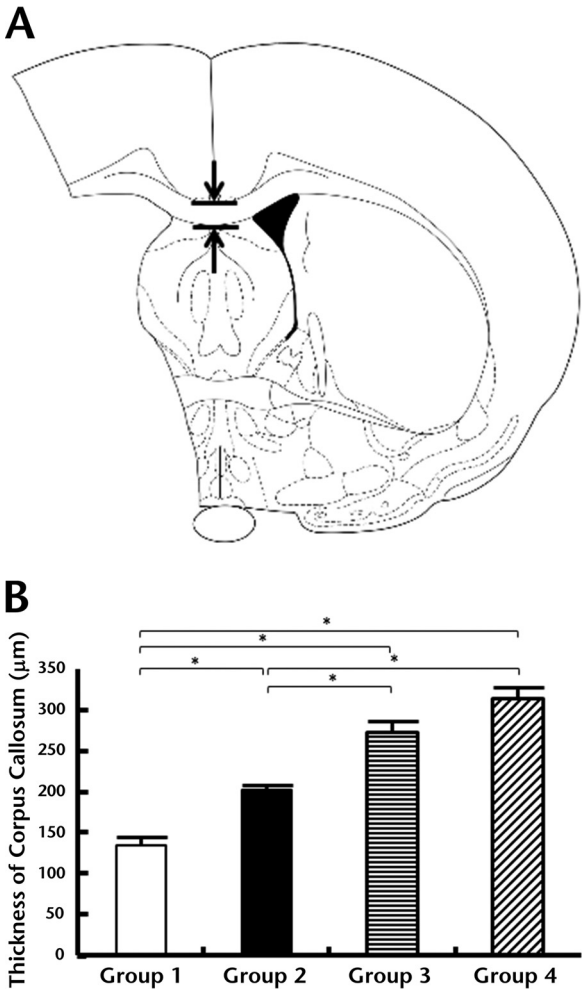


**Figure 3.**
(A) Corpus callosum thickness was measured at the midline with T2-weighted images. (B) Quantitative results of corpus callosum thickness. Group 1=vehicle only, group 2=vehicle + exercise, group 3=mesenchymal stem cells (MSCs) only, and group 4=MSCs + exercise. * $P<.01$.

### Behavioral Analysis

To assess behavioral performance in the 4 experimental groups, the LPT was performed at days 1, 14, and 35 after MCAO. Before MCAO, LPT neurological scores were similar among all animals, having a value of about 16 (N=50). As tested immediately before cell infusion, there were equal deficits in LPT score among the 4 experimental groups, with values of about 2.0 (Fig. 4). In groups 3 and 4, the improvements in scores were greater over the recovery time course (14 and 35 days) compared with the non–MSC-infused groups. Groups 2 and 3 showed improved LPT scores compared with group 1 at days 14 and 35, but group 3 showed greater improvement. Group 4 achieved the highest score at 35 days compared with other 3 experimental groups, indicating that the combination of exercise and MSC treatment was more effective than either alone. These results are summarized in Figure 4.

### Correlation Analysis of Anatomical Change and Behavioral Function

To determine whether there was a positive relationship between behavioral function and both the density of synaptic puncta and the thickness of CC obtained from each animal from the intact group and all experimental groups, we plotted the LPT scores as a function of the synaptic puncta (Fig.5A) and the thickness of CC (Fig. 5B). A Pearson correlation analysis indicated a significant positive correlation of LPT scores versus density of synaptic puncta (Pearson $r=.92$, $P<.01$, n=29) and the thickness of CC (Pearson $r=.78$, $P<.01$, n=29). The linear regression of these data is shown by the solid line in Fig. 5A ($y = 0.0783x - 0.0887$) and Fig 5B ($y = 19.423x + 80.46$). Thus, the density of anatomical changes evaluated synaptogenesis, and the CC thickness was correlated with the degree of functional improvements. Taken together, these results suggest that the degree of behavioral improvement might be associated with the combined therapy, whose (group 4) anatomical changes were greatest compared with the other experimental groups and greater in the MSC groups (groups 3 and 4) compared with the non-MSC groups (groups 1 and 2).

## Discussion

The present study demonstrated that combined therapy of daily rehabilitation and intravenous infusion of MSCs 6 hours after permanent MCAO in the rat resulted greater reduction in infarction volume, improvement in behavioral performance, and facilitation of synaptogenesis compared with either MSC or rehabilitation alone. These results are consistent with previous studies showing beneficial effects of bone marrow cell transplantation in experimental cerebral ischemic models.[3,5,20,22] Although individually both rehabilitation and MSC therapy showed efficacy, the effects were greater in the group 4.

The mechanisms for the functional recovery in stroke are not clear, but studies have demonstrated remapping of cortical function, redistribution of neuronal activity, cortical reorganization, and rewiring of surviving circuits in experimental cerebral infarction models and clinical studies in patients with stroke that may contribute to observed spontaneous recovery.[33–36] This cortical plasticity after cortical infarction has been attributed to axonal sprouting and synaptogenesis, which may be in response to the facilitation of neurotrophic factors. In the present study, we observed that combination therapy with daily

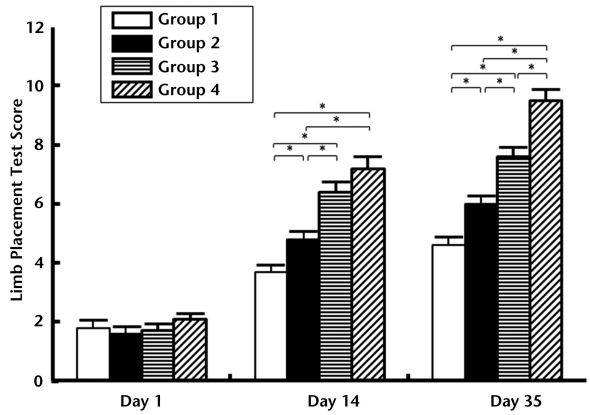


**Figure 4.**
Assessment of behavioral function with the Limb Placement Test (LPT). One day after middle cerebral artery occlusion (MCAO), there was no statistical difference in LPT score among the 4 experimental groups. From day 14 on, groups 3 and 4 showed greater functional improvement compared with groups 1 and 2, but group 4 showed more improvement compared with group 3 at 35 days after MCAO. Group 1=vehicle only, group 2=vehicle + exercise, group 3=mesenchymal stem cells (MSCs) only, and group 4=MSCs + exercise. * $P<.01$.

rehabilitation and intravenous infusion of MSCs significantly increased the expression of synaptophysin in the motor cortex in the infarcted hemisphere, suggesting the facilitation of neuronal remodeling in the cortex. The increased thickness of the CC may be from preservation of transcortical projections or possibly from enhanced structural rewiring between the hemispheres, either of which might contribute to functional improvement of the ischemic stroke. Distribution of the intravenously infused GFP-MSCs observed in the motor cortex in the infarcted hemisphere might contribute to the cortical plasticity because the MSCs could potentially signal via trophic factors to cortical neurons.[37]

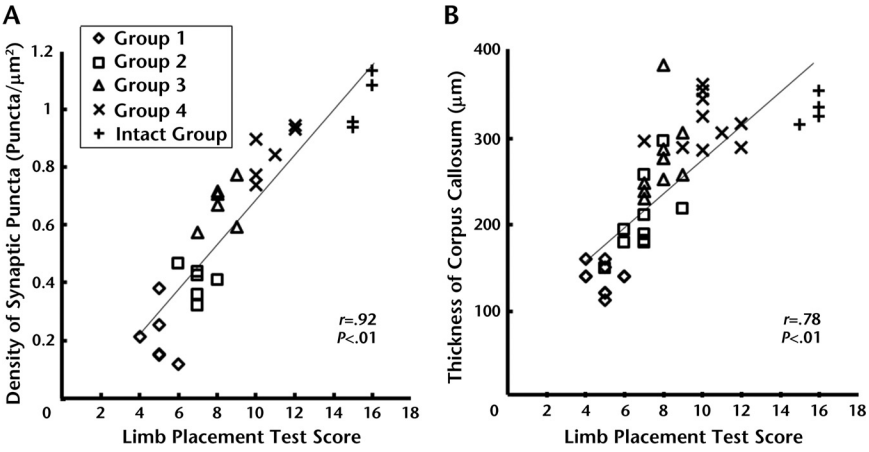


**Figure 5.**
(A) The increased expression of synaptic marker protein correlated well with increased Limb Placement Test (LPT) scores, as illustrated by positive correlations between the density of synaptic puncta and LPT scores ($r=.92$, $P<.01$). (B) The increased thickness of corpus callosum (CC) correlated well with increased LPT scores, as illustrated by positive correlations between the thickness of CC and LPT scores ($r=.78$, $P<.01$). Group 1=vehicle only, group 2=vehicle + exercise, group 3=mesenchymal stem cells (MSCs) only, and group 4=MSCs + exercise.

Andres et al[38] reported that rats after MCAO with direct transplantation of human neural stem cells into the infarcted cortex had increased corticostriatal and corticothalamic projections and corticospinal axonal rewiring from the contralesional cortex, as evidenced by anterograde axonal tracer methods. It is conceivable that other tracts, including transcallosal corticospinal and other extrapyramidal pathways, could be enhanced by the trophic influences of the infused MSCs and lead to increased functional activity. Clearly, more detailed anatomical studies will be needed to identify the enhanced neuronal circuits in the central nervous system after MSC therapy with daily rehabilitation and intravenous infusion of MSCs.

Cell-based therapeutic approaches are being considered for a number of neurological diseases, including stroke.[8,9] Suggested mechanisms include remodeling of neural circuits, synaptogenesis, reduction of inflammatory infiltration and demyelination, and elevation of trophic factors that may be neuroprotective.[23,39] We reported in an initial phase I/II clinical trial in patients with stroke following intravenous infusion of autologous human MSCs that all patients received formal rehabilitation (physical therapy, occupational therapy, and speech therapy), resulting in a trend of improvement in patients with stroke.[9] A cell-based therapy with rehabilitation may have the advantage of facilitating multiple therapeutic effects by MSCs at various sites and times within the lesion, as the MSCs respond to a particular pathological microenvironment. In summary, our results indicate that combined therapy of daily rehabilitation and intravenous infusion of MSCs improved functional outcome in a rat MCAO model.

In conclusion, we examined whether combination therapy of daily rehabilitation and intravenous infusion of MSCs improves functional outcome in a rat stroke model. Functional recovery was observed in the MSC-treated groups with greatest recovery in the group that received combined therapy and MSC infusion. Reduction of stroke volume and induced synaptogenesis might contribute to the functional improvements.

Dr M. Sasaki, Dr Kocsis, and Dr Honmou provided concept/idea/research design and writing. Dr Sasaki, Dr Kataoka-Sasaki, Dr Nakazaki, Dr T. Namioka, and Dr A. Namioka provided data collection. All authors provided data analysis. Dr M. Sasaki provided project management. Dr M. Sasaki, Dr Onodera, and Dr Honmou provided fund procurement. Dr M. Sasaki and Dr Honmou provided facilities/equipment. Dr Ishiai and Dr Honmou provided institutional liaisons. Dr Kocsis provided consultation (including review of manuscript before submission).

This work was supported, in part, by JSPS KAKENHI grant numbers 26350618, 25462226, 24592138, 26462214, and 15K10365; the AMED Translational Research Network Program (B51) and the RR&D Service of Department of Veterans Affairs; the VA SCI Translational Collaborative Consortium for Regenerative Medicine; and the NMSS (grant RG2135 to Dr Kocsis). The authors are thankful to the National BioResource Project–Rat (http://www.anim.med.kyoto-u.ac.jp/NBR/) for providing rat strains (W-Tg[CAG-GFP]184Ys).

DOI: 10.2522/ptj.20150504

## References

1 Chen J, Li Y, Wang L, Lu M, et al. Therapeutic benefit of intracerebral transplantation of bone marrow stromal cells after cerebral ischemia in rats. *J Neurol Sci.* 2001;189:49–57.

2 Li Y, Chen J, Chen XG, et al. Human marrow stromal cell therapy for stroke in rat: neurotrophins and functional recovery. *Neurology*. 2002;59:514–523.

3 Iihoshi S, Honmou O, Houkin K, et al. A therapeutic window for intravenous administration of autologous bone marrow after cerebral ischemia in adult rats. *Brain Res.* 2004;1007:1–9.

4 Nomura T, Honmou O, Harada K, et al. I.v. infusion of brain-derived neurotrophic factor gene-modified human mesenchymal stem cells protects against injury in a cerebral ischemia model in adult rat. *Neuroscience*. 2005;136:161–169.

5 Honma T, Honmou O, Iihoshi S, et al. Intravenous infusion of immortalized human mesenchymal stem cells protects against injury in a cerebral ischemia model in adult rat. *Exp Neurol.* 2006;199:56–66.

6 Chen X, Li Y, Wang L, et al. Ischemic rat brain extracts induce human marrow stromal cell growth factor production. *Neuropathology*. 2002;22:275–279.

7 Parr AM, Tator CH, Keating A. Bone marrow-derived mesenchymal stromal cells for the repair of central nervous system injury. *Bone Marrow Transplant.* 2007;40:609–619.

8 Honmou O, Onodera R, Sasaki M, et al. Mesenchymal stem cells: therapeutic outlook for stroke. *Trends Mol Med.* 2012;18:292–297.

9 Honmou O, Houkin K, Matsunaga T, et al. Intravenous administration of auto serum-expanded autologous mesenchymal stem cells in stroke. *Brain*. 2011;134:1790–1807.

10 Kim MW, Bang MS, Han TR, et al. Exercise increased BDNF and trkB in the contralateral hemisphere of the ischemic rat brain. *Brain Res.* 2005;1052:16–21.

11 Pagnussat AS, Simao F, Anastacio JR, et al. Effects of skilled and unskilled training on functional recovery and brain plasticity after focal ischemia in adult rats. *Brain Res.* 2012;1486:53–61.

12 Yu K, Wu Y, Hu Y, et al. Neuroprotective effects of prior exposure to enriched environment on cerebral ischemia/reperfusion injury in rats: the possible molecular mechanism. *Brain Res.* 2013;1538:93–103.

13 Wang D, Ichiyama RM, Zhao R, et al. Chondroitinase combined with rehabilitation promotes recovery of forelimb function in rats with chronic spinal cord injury. *J Neurosci.* 2011;31:9332–9344.

14 Dobkin BH, Dorsch A. New evidence for therapies in stroke rehabilitation. *Curr Atheroscler Rep.* 2013;15:331.

15 Richards CL, Malouin F, Wood-Dauphinée S, et al. Task-specific physical therapy for optimization of gait recovery in acute stroke patients. *Arch Phys Med Rehabil.* 1993;74:612–620.

16 Hesse S, Bertelt C, Jahnke MT, et al. Treadmill training with partial body weight support compared with physiotherapy in nonambulatory hemiparetic patients. *Stroke.* 1995;26:976–981.

17 Cotman CW, Berchtold NC. Exercise: a behavioral intervention to enhance brain health and plasticity. *Trends Neurosci.* 2002;25:295–301.

18 Kim S, Honmou O, Kato K, et al. Neural differentiation potential of peripheral blood- and bone-marrow-derived precursor cells. *Brain Res.* 2006;1123:27–33.

19 Ukai R, Honmou O, Harada K, et al. Mesenchymal stem cells derived from peripheral blood protects against ischemia. *J Neurotrauma*. 2007;24:508–520.

20 Onda T, Honmou O, Harada K, et al. Therapeutic benefits by human mesenchymal stem cells (hMSCs) and Ang-1 gene-modified hMSCs after cerebral ischemia. *J Cereb Blood Flow Metab.* 2008;28:329–340.

21 Sasaki M, Honmou O, Kocsis JD. A rat middle cerebral artery occlusion model and intravenous cellular delivery. *Methods Mol Biol.* 2009;549:187–195.

22 Liu H, Honmou O, Harada K, et al. Neuroprotection by PIGF gene-modified human mesenchymal stem cells after cerebral ischaemia. *Brain*. 2006;129:2734–2745.

23 Sasaki M, Radtke C, Tan AM, et al. BDNF-hypersecreting human mesenchymal stem cells promote functional recovery, axonal sprouting, and protection of corticospinal neurons after spinal cord injury. *J Neurosci.* 2009;29:14932–14941.

24 Zheng W, Honmou O, Miyata K, et al. Therapeutic benefits of human mesenchymal stem cells derived from bone marrow after global cerebral ischemia. *Brain Res.* 2010;1310:8–16.

25 Osaka M, Honmou O, Murakami T, et al. Intravenous administration of mesenchymal stem cells derived from bone marrow after contusive spinal cord injury improves functional outcome. *Brain Res.* 2010;1343:226–235.

26 Davies SJA, Shih C-H, Noble M, et al. Transplantation of specific human astrocytes promotes functional recovery after spinal cord injury. *PLoS One*. 2011;6:e17328.

27 Alexanian AR, Fehlings MG, Zhang Z, Maiman DJ. Transplanted Neurally modified bone marrow-derived mesenchymal stem cells promote tissue protection and locomotor recovery in spinal cord injured rats. *Neurorehabil Neural Repair*. 2011;25:873–880.

28 Komatsu K, Honmou O, Suzuki J, et al. Therapeutic time window of mesenchymal stem cells derived from bone marrow after cerebral ischemia. *Brain Res.* 2010;1334:84–92.

29 Neumann-Haefelin T, Kastrup A, de Crespigny A, et al. Serial MRI after transient focal cerebral ischemia in rats: dynamics of tissue injury, blood-brain barrier damage, and edema formation. *Stroke.* 2000;31:1965–1972; discussion 1972–1973.

30 Paxinos G, Watson C. *The Rat Brain in Stereotaxic Coordinates*. 4th ed. San Diego, CA: Academic Press; 1998.

31 Ohlsson AL, Johansson BB. Environment influences functional outcome of cerebral infarction in rats. *Stroke.* 1995;26:644–649.

32 Ujike H, Takaki M, Kodama M, Kuroda S. Gene expression related to synaptogenesis, neuritogenesis, and MAP kinase in behavioral sensitization to psychostimulants. *Ann. NY Acad. Sci.* 2002;965:55–67.

33 Dancause N, Barbay S, Frost SB, et al. Extensive cortical rewiring after brain injury. *J Neurosci.* 2005;25:10167–10179.

34 Nudo RJ. Postinfarct cortical plasticity and behavioral recovery. *Stroke*. 2007;38:840–845.

35 Murphy TH, Corbett D. Plasticity during stroke recovery: from synapse to behaviour. *Nat Rev Neurosci.* 2009;10:861–872.

36 Benowitz LI, Carmichael ST. Promoting axonal rewiring to improve outcome after stroke. *Neurobiol Dis.* 2010;37:259–266.

37 Ren G, Chen X, Dong F, et al. Concise review: mesenchymal stem cells and translational medicine: emerging issues. *Stem Cells Transl Med.* 2012;1:51–58.

38 Andres RH, Horie N, Slikker W, et al. Human neural stem cells enhance structural plasticity and axonal transport in the ischaemic brain. *Brain*. 2011;134:1777–1789.

39 Takayanagi A, Sasaki M, Kataoka-Sasaki Y, et al. Intravenous preload of mesenchymal stem cells rescues erectile function in a rat model of cavernous nerve injury. *J Sex Med.* 2015; 12:1713–1721.