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Biochemical and Biophysical Research Communications 368 (2008) 151–156
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Delayed injection of neural progenitor cells improved spatial learning dysfunction after cerebral ischemia

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Received 18 December 2007
Available online 28 January 2008

Abstract

Although stem cells are likely to improve neurological deficits seen after cerebral ischemia, the effects of neural progenitor cells (NPCs) on cerebral ischemia-induced learning dysfunction remain to be clarified. We tested whether the delayed injection of exogenous NPCs could prevent learning dysfunction after cerebral ischemia. Cerebral ischemia was produced by the injection of microspheres into the right hemisphere of each rat. Injection of NPCs obtained from green fluorescent protein transgenic rats into the hippocampus on Day 7 after the induction of cerebral ischemia improved the modified neurological severity score and reduced the prolongation of the escape latency seen in the water maze task. A few of the injected NPCs were positive for mature neuronal markers. In addition, the injected NPCs expressed BDNF on Day 28 after cerebral ischemia. Thus, the exogenous NPCs delivered by injection could act as a source of neurotrophic factors and prevent cerebral ischemia-induced learning dysfunction.

Keywords: Brain; Cerebral ischemia; Microsphere embolism; Learning and memory; Water maze test; Neural progenitor cells

Neurogenesis in the central nervous system is accelerated after cerebral ischemia. Newly generated cells after cerebral ischemia proliferate [1–3] and migrate to the site of injury in the brain [4,5]. As these cells express markers of mature neurons after their migration [4], they are likely to have a potential to repair brain injuries induced by cerebral ischemia. However, the majority of them fail to survive [4]. Therefore, the repair of ischemic brain injuries by endogenous neural progenitor cells (NPCs) would appear to be unlikely.

It was earlier reported that the injection of stem cells induces functional recovery, such as improvement of neurological deficits and motor coordination dysfunction after cerebral ischemia [6,7]. However, it remains to be clarified whether neural NPCs can improve learning and memory dysfunction after cerebral ischemia. Microsphere-induced cerebral embolism in rats induces widespread formation of small permanent emboli and multiple infarct areas in the brain and severe spatial learning dysfunction as assessed by the water maze task [8]. Therefore, the microsphere embolism model is considered to mimic focal ischemia-induced human stroke [9] or multi-infarct dementia [10].

In some cases, the microenvironment during the acute phase after tissue injury is insufficient to allow the administrated cells to ameliorate cell injury [11]. In the present study, we investigated whether the delayed injection of exogenous NPCs could improve spatial learning dysfunction, as assessed by using a water maze test, after severe cerebral ischemia. To determine the phenotype of the injected NPCs, we prepared NPCs from green fluorescent protein (GFP) transgenic rats by using the neurosphere method and injected them into the hippocampus after microsphere embolism. Our findings demonstrate that the exogenous NPCs delivered by delayed injection could act

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doi:10.1016/j.bbrc.2008.01.084

as a source of neurotrophic factors and prevent cerebral ischemia-induced learning dysfunction.

Materials and methods

Animal model of microsphere-embolized (ME) rat. Male Wistar rats weighing 220–260 g (Charles River Japan Inc., Atsugi, Japan) were used. The animals had free access to food and water according to the National Institute of Health Guide for the Care and Use of Laboratory Animals and the Guidance for Experimental Animal Care issued by the Prime Minister's Office of Japan. All experiments were approved by the Committee of Animal Care and Welfare of Tokyo University of Pharmacy and Life Sciences.

Microsphere-induced cerebral embolism was performed by the method described previously [8]. In brief, 700 microspheres (47.5 ± 0.5 μm in diameter, Perkin-Elmer Life Science), suspended in 20% dextran solution, were injected into the right internal carotid artery through a cannula. The rats that underwent a sham operation received the same volume of vehicle without microspheres.

On Day 7 after surgery, the serum level of IL-6 was determined by using a BD™ CBA Rat IL-6 Flex Set (BD Biosciences, San Diego, CA, USA) according to the manufacturer's protocol.

Neural progenitor cell cultures. Neural progenitor cells (NPCs) were prepared from gestational day-14 fetal green fluorescent protein (GFP) transgenic rats by the method described previously [12–14]. The GFP transgenic rats [Wistar-TgN(CAG-GFP)184ys] used in this study were provided by the National Bio Resource Project for the Rat in Japan (Kyoto, Japan). The origin and characteristics of these transgenic rats were previously described [15]. Cells were seeded at a density of 50,000 cells/cm^2 into non-treated flasks containing N-2 plus medium supplemented with 20 ng/ml epidermal growth factor (EGF) and 20 ng/ml basic fibroblast growth factor (b-FGF) (growth medium). NPCs were grown in culture as free-floating neurospheres, and 80% of the medium was replaced with new growth medium on day 4. Neurospheres cultured for 7 days *in vitro* were used for the experiments. Immunostaining of cultured neurospheres was done by the method described previously [13].

Bromodeoxyuridine labeling. Two 5-bromo-2'-deoxyuridine (BrdU, Sigma-Aldrich, St. Louis, MO, USA) injection paradigms were used in the present study. In the first series of experiments, rats were administered a single dose of BrdU (50 mg/kg) intraperitoneally at 10 min, 2 days, 6 days, 13 days or 20 days after the embolism. Twenty-four hours after the administration, the animals were perfused transcardially with 4% paraformaldehyde under deep anesthesia. This experiment reveals an index of the rate of generation of cells at a specific time point after the embolism. In the second experiment, rats received intracerebroventricular administration of BrdU on Day 7 after the embolism. BrdU (100 μg/kg) was infused into left ventricle at the rate of 0.5 μL/min using a microsyringe pump. This experiment discloses the phenotype and migration pattern of proliferative cells in the SVZ.

Injection of NPCs. Neurospheres were resuspended in N-2 plus medium to a final concentration of 5.0×10^4 cells/μl. The cell suspension (2 μl) was injected via a 27-gauge needle implanted 3.7 mm lateral and 3.9 mm posterior to the bregma and at a depth of 3.6 mm from the cortical surface. Cells were injected at 2 μl/min while withdrawing the needle in 100-μm increments every 10 s. Vehicle was injected in a similar manner as were the NPCs.

Behavioral testing. A battery of behavioral tests was performed on the basis of modified Neurological Severity Scores (mNSS) [6] by an investigator who was blinded to the experimental groups. Neurological function was graded on a scale of 0–18 (normal score, 0; maximal deficit score, 18).

Water maze test. The water maze test was performed according to method described previously [8]. The acquisition test was started on Day 12 after surgery. Animals were tested in the water maze by using a 3 trials/day regimen for 3 days. Data collection was automated for each trial with a behavioral tracing analyzer (WaterMaze ver2.6; Neuroscience, Tokyo, Japan).

The retention test was performed on Days 21 and 28 after surgery to determine whether the animals could retain the spatial navigation ability in the hidden platform test. The regimen and starting point used for this task were the same as those for the acquisition test on Day 14.

Histological assessments. At various times after surgery, the rats were perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer. Immunohistochemical detection was performed by the method described previously [2].

The primary antibodies used were rat monoclonal anti-GFP (Nacalai Tesque, Kyoto, Japan), rat monoclonal anti-BrdU (Oxford Biotechnology, Oxford, UK), mouse monoclonal anti-nestin (BD Bioscience, Franklin, NJ, USA), mouse monoclonal anti-NeuN (Chemicon), rabbit polyclonal anti-glial fibrillary acidic protein (GFAP; DAKO, Carpinteria, CA, USA), mouse monoclonal anti-microtubule-associated protein 2 (MAP-2, Sigma), and rabbit polyclonal anti-BDNF (Chemicon) antibodies. The secondary antibodies used were fluorescein isothiocyanate-conjugated donkey anti-rat IgG (Jackson Immunoresearch, West Grove, PA, USA), Cy3-conjugated goat anti-mouse IgG (Amersham, Buckinghamshire, UK), and Cy3-conjugated donkey anti-rabbit IgG (Jackson Immunoresearch). Fluorescence was detected by using an Olympus fluorescence microscope (BX-52, Olympus, Tokyo, Japan) or a KEYENCE BZ-8000 (KEYENCE, Osaka, Japan).

Statistical analysis. The results were expressed as means ± standard error of the mean (SEM). Statistical comparison among multiple groups was evaluated by analysis of variance (ANOVA) followed by Fisher's protected least significant difference (PLSD) as a *post hoc* test. *P*-values of less than 0.05 were considered significant.

Results

Cell proliferation and differentiation

As microsphere embolism-induced severe tissue damage to the hippocampus, we determined cell proliferation after microsphere embolism in the subventricular zone (SVZ) of the lateral ventricles ($n = 4$ each time point). Increases in BrdU incorporation were detected on Days 3, 7, and 14 after the embolism (Fig. 1A). Maximally, a 2.4-fold increase in the number of BrdU-labeled cells compared with the number for sham-operated animals was seen on Day 7 after the embolism (Fig. 1A and B).

To determine the migration and changes in the number of BrdU-labeled cells, BrdU was injected on Day 7 after the embolism into the left cerebral ventricle. We used the number of BrdU-labeled cells in the SVZ at 2 h after the injection of BrdU as the initial value (150.5 ± 4.6 cells/SVZ; Fig. 2A). The number of BrdU-labeled cells in the SVZ decreased with time (Fig. 2A and C). Quantitative analysis revealed a decrease in the number of BrdU-labeled cells in the SVZ on Days 7, 14, 21, and 28 after BrdU injection compared with the initial value (Fig. 2A). In contrast, the number of BrdU-labeled cells in the striatum increased on Days 1, 3, and 7 after BrdU injection compared with the initial value (65.6 ± 19.8 cells/striatum; Fig. 2B and C). BrdU-labeled cells in the SVZ expressed nestin, a neural progenitor marker, and doublecortin (DCX), a microtubule-associated protein found in migrating immature neurons, on Day 3 after the injection of BrdU (Fig. 2D) and throughout the remainder of the experiment, whereas they did not express MAP2, a neuronal marker, or GFAP, an astrocyte marker, in the SVZ (not shown). On Day 28 after the injection of BrdU, a few BrdU-labeled cells expressed MAP2 (Fig. 2D), whereas GFAP was not

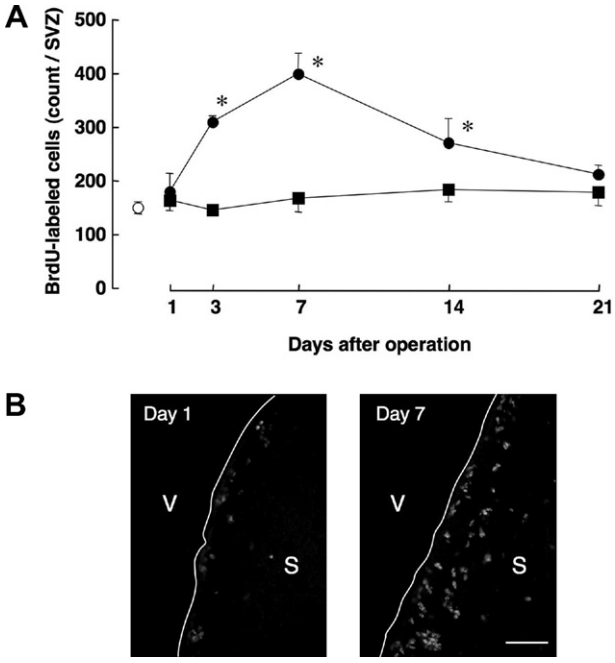

Fig. 1. Microsphere embolism-induced cell proliferation in the SVZ. (A) Number of BrdU-labeled cells in the SVZ of non-operated naïve (open circle), sham-operated (closed squares), and ME rats (closed circles) on Days 1 to 21 after surgery. Values are presented as means ± SEM, $n = 4$ each. *Significant difference from sham-operated rats ($p < 0.05$). (B) Representative images of BrdU-labeled cells in the SVZ of ME rats on Days 1 and 7. V, ventricle. S, striatum. Scale bar, 50 μm.

expressed in the BrdU-labeled cells in the striatum (Fig. 2D).

There were no significant differences in the serum level of IL-6 on Day 7 among non-operated naïve (249.4 ± 16.5 pg/ml), sham-operated (283.6 ± 11.2 pg/ml), and ME (276.4 ± 21.2 pg/ml) rats ($n = 3$ each).

Effect of injection of NPCs on modified Neurological Severity Scores (mNSS) and the water maze test

We next examined the effects of exogenous injection of NPCs on sustained cerebral ischemia-induced injury. NPCs that had been isolated from GFP transgenic rats were injected into the hippocampal CA2 region on Day 7 after the embolism.

At first, we examined the mNSS, calculated based on a series of motor sensory, reflex, and balance tests [6]. The score of vehicle-treated ME rats was 8.3 ± 0.5 points on Day 14 after surgery. Then, the score remained at that value up to 28 days. There was a significant difference in mNSS by group [$F(1,16) = 18.3$, $p < 0.001$, $n = 9$] and by days [$F(4,64) = 63.1$, $p < 0.0001$, $n = 9$]. The group by day interaction was significant ($p < 0.0001$, $n = 9$). The scores of NPC-injected ME rats significantly decreased on Days 14, 21, and 28 ($p < 0.001$, $n = 9$ each; Fig. 3A).

We next measured the escape latency of the acquisition test in the water maze task on Days 12–14 after the embolism (Fig. 3B). There was a significant difference in the escape latency by group [$F(2,24) = 2.1$, $p < 0.01$, $n = 9$] and by days [$F(8,192) = 10.8$, $p < 0.0001$, $n = 9$]. The escape latency of the vehicle-treated ME rats was lengthened significantly compared with that of the sham-operated

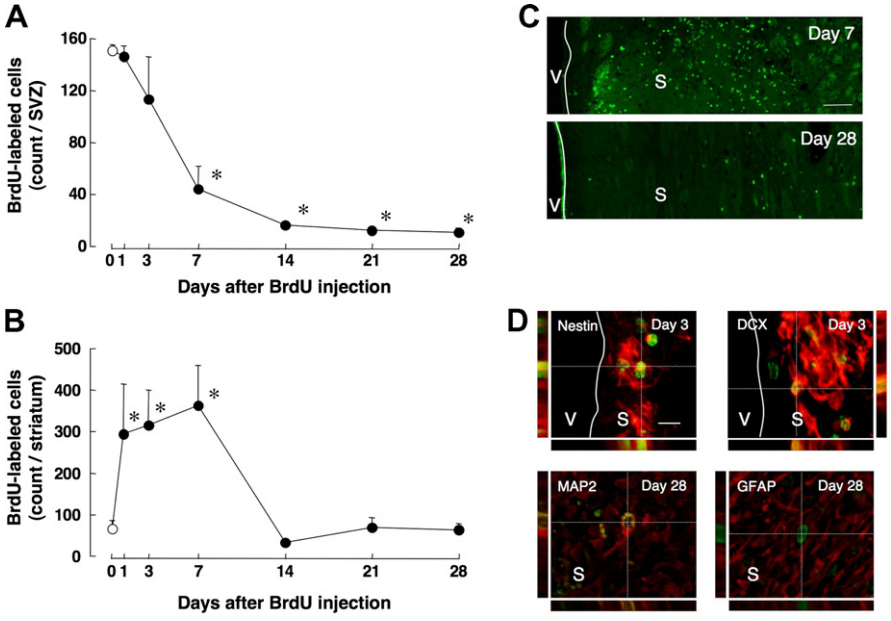

Fig. 2. Microsphere embolism-induced cell migration and differentiation in the striatum. (A and B) The numbers of BrdU-labeled cells in the SVZ (A) and striatum (B) of microsphere embolized rats are shown. BrdU was infused into the left ventricle on Day 7 after microsphere embolism. The number of BrdU-labeled cells at 2 h after the infusion of BrdU was used as the initial value (open circle). Values are presented as means ± SEM, $n = 4$ each. *Significant difference from the initial value ($p < 0.05$). (C) Representative images of BrdU-labeled cells in the striatum of ME rats on Days 7 and 28 after BrdU infusion. V, ventricle. S, striatum. Scale bar, 100 μm. (D) Images of double staining for BrdU (green) and Nestin (red), DCX (red), MAP2 (red) or GFAP (red) on Days 3 and 28 after the infusion of BrdU. V, ventricle. S, striatum. Scale bar, 10 μm.

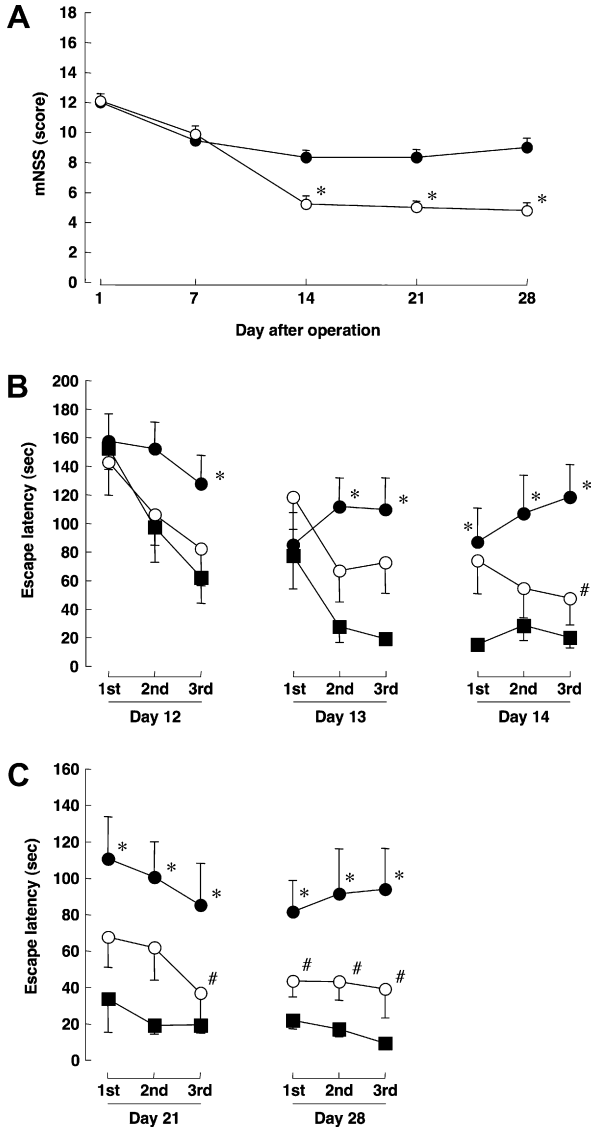

Fig. 3. (A) Time course of change in mNSS of NPC-injected (open circles) and vehicle-treated (closed circles) ME rats. (B and C) Changes in the escape latency of the acquisition (B) and retention (C) tests of the water maze test for vehicle-treated (closed circles) and NPC-injected (open circles) ME rats, and for sham-operated (closed squares) rats. Each value is presented as means ± SEM of 9 animals. *Significant difference from the sham-operated rats ($p < 0.05$). #Significant difference from the vehicle-treated ME rats ($p < 0.05$).

rats at the third trial of Day 12 and from the second trial of Day 13 to the third trial of Day 14 ($p < 0.05$, $n = 9$ each). The injection of NPCs attenuated the prolonged escape latency of the vehicle-treated ME rats at the third trial on Day 14 ($p < 0.05$, $n = 9$; Fig. 3B).

The retention test was performed on Days 21 and 28 with the same regimen as that on Day 14 (Fig. 3C). There was a significant difference in the escape latency by group on Day 21 [$F(2,24) = 9.7$, $p < 0.001$, $n = 9$]. The group by day interaction was not significant ($p = 0.95$, $n = 9$). On Day 28, there was a significant difference in the escape latency by group [$F(2,24) = 8.7$, $p < 0.01$, $n = 9$]. Injection of NPCs attenuated the prolonged escape latency of the vehicle-treated ME rats from the third trials on Day 21 to the third trials on Day 28 ($p < 0.05$, n = 9 each; Fig. 3C). The swimming speed in the water maze task was similar in all groups examined.

Histological analysis

A few of the injected NPCs (GFP-positive cells) expressed NeuN (Fig. 4A), but not GFAP (Fig. 4B) on Day 28 after the injection of NPCs. Neurospheres obtained from GFP-transgenic rats expressed BDNF (Fig. 4C). In addition, BDNF was expressed within GFP-positive NPCs on Day 28 after the injection (Fig. 4D).

Discussion

The maximum incorporation of BrdU was seen on Day 7 after the embolism, suggesting that cell proliferation was accelerated most on Day 7 after the embolism. However, only a small number of cells that proliferated at this time differentiated into cells that expressed MAP-2. In addition, the number of BrdU-labeled cells on Day 7 after the embolism decreased with time. These results raise the possibility that endogenous proliferating cells induced by microsphere embolism are insufficient for maintenance of brain function under pathological conditions such as cerebral ischemia. Therefore, supplementation with exogenous NPCs may lead to functional recovery. In this sense, we demonstrated that the injection of NPCs into the hippocampus improved the mNSS and spatial learning dysfunction after microsphere embolism.

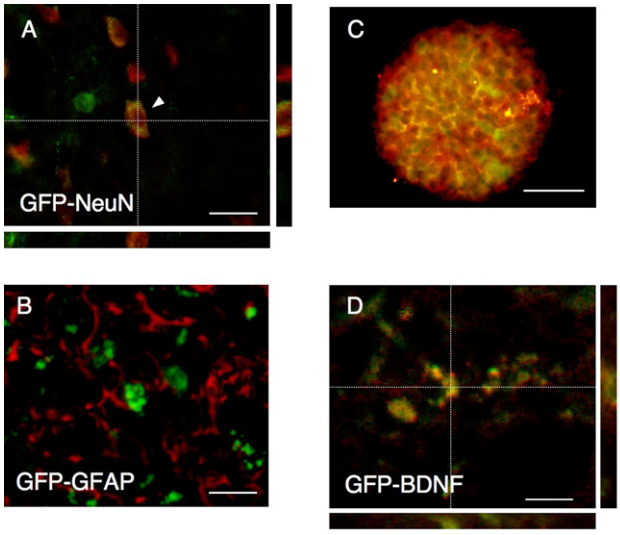

Fig. 4. Images obtained by double staining for GFP (green) and NeuN (red, A), GFAP (red, B) or BDNF (red, C and D) in the hippocampus of microsphere embolized rats on Day 28 after the injection (A, B, and D) or in a neurosphere in growth medium on Day 7 (C). Images were combined to produce a three-dimensional reconstruction. Scale bar, 10 μm (A, B, and D) and 50 μm (C).

Accumulating evidence indicates that there is a therapeutic time window for successful transplantation [11]. Indeed, the mNSS was not ameliorated by the injection of NPCs at 10 min after the embolism (data not shown). In the present study, it is not clear why the injection of NPCs at the acute phase of microsphere embolism was ineffective in lowering the mNSS. An acute phase is not likely to be appropriate for therapeutic transplantation, since severe inflammation occurs around the injured site during this phase. The levels of inflammatory cytokines, which provoke neurotoxicity, increase during the acute phase of spinal cord injury [16]. Various cytokines, such as IL-1β, IL-6, and tumor necrosis factor-α, are also induced by cerebral ischemia. These inflammatory cytokines have been suggested to play a role in contributing to the ischemic injury, although it was reported that endogenous IL-6 plays a key role in preventing damaged neurons in the acute phase of middle cerebral artery occlusion and reperfusion [17]. As the level of IL-6 on Day 7 after the embolism was comparable to that of sham-operated control animals, we injected NPCs on Day 7 into the hippocampus to avoid an inappropriate environment for NPCs as much as possible. Therefore, NPCs injected on Day 7 after the embolism might not be adversely affected by ischemia-induced inflammatory cytokines and consequently might be able to contribute to the functional recovery.

Although the mechanisms by which injected NPCs induce functional recovery after an embolism have not been fully elucidated, intravenous injection of human bone marrow stromal cells enhanced recovery of the mNSS after permanent middle cerebral artery occlusion in rats, possibly through the induction of growth factors [18]. It was also reported that neurotrophic factors were secreted from cultured NPCs [19]. Furthermore, it has been suggested that neurotrophic growth factors are involved in the proliferation, differentiation, and survival of NPCs [20] and in functional recovery after cerebral ischemia, possibly through stimulation of progenitor cells [21]. Therefore, neurotrophic factors are likely to play a pivotal role in self-repair processes for NPCs and inducing functional recovery.

Most importantly, an improvement of escape latency in the water maze test was found in animals given NPCs even on Day 7 after the embolism. Microsphere embolism induces severe and sustained injury. Therefore, even though a small number of GFP-positive injected cells expressed markers of mature neurons, it is unlikely that some of these NPCs made appropriate connections with endogenous neurons within days after the injection to afford the improvement noted. In the central nervous system, BDNF plays a pivotal role in learning and memory function [22]. BDNF not only reduced infarct size after transient focal cerebral ischemia [23], but also improved cognitive functions after transient global cerebral ischemia [24]. We demonstrated that cultured NPCs were positive for BDNF and that NPCs injected into the ischemic brain expressed BDNF protein even on Day 28 after the injection. Therefore, the injected NPCs might have the ability to secrete neurotrophic factors, such as BDNF and/or stimulate endogenous cells in the ischemic brain to release BDNF that might be associated with improved spatial learning function. Taken together, our data suggest that the injected NPCs served as a source of trophic factor production in response to ischemic brain injury rather than as one of mature neurons connecting to surrounding endogenous neurons.

In conclusion, the injection of NPCs even on Day 7 after the embolism improved the mNSS and spatial learning dysfunction. Although the present study did not obviously reveal that BDNF produced by the injected NPCs directly induced functional recovery, such cells might be a source of neurotrophic factors for improving ischemic brain injury, including learning and memory dysfunction.

Acknowledgments

This work was supported in part by the Promotion and Mutual Aid Corporation for Private School of Japan.

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