available at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/brainres

BRAIN RESEARCH

Research Report

Neuroprotective effect of grafting *GDNF* gene-modified neural stem cells on cerebral ischemia in rats

B. Chen[a], *X.-Q. Gao*[a], *C.-X. Yang*[a], *S.-K. Tan*[a], *Z.-L. Sun*[a], *N.-H. Yan*[b], *Y.-G. Pang*[a], *M. Yuan*[a], *G.-J. Chen*[a], *G.-T. Xu*[d], *K. Zhang*[c,d], *Q.L. Yuan*[c,d,*]

[a]*Luzhou Medical College, Department of Neurobiology, 646000, China*
[b]*Medicine Laboratory of Ophthalmology Molecular Genetics, West China Hospital, Sichuan University, Chengdu 610041, China*
[c]*Department of Anatomy and Neurobiology, Tongji University School of Medicine, Shanghai 200092, China*
[d]*Department of Regenerative Medicine, Tongji University School of Medicine, Shanghai 200092, China*

ARTICLE INFO

Article history:
Accepted 28 May 2009
Available online 9 June 2009

Keywords:
Neural stem cell
Transplantation
Gene therapy
Neuroprotection
Glial cell line-derived neurotrophic factor
NT-3
Brain-derived neurotrophic factor
Stroke
Synaptic protein

ABSTRACT

Previous studies indicated the beneficial effects of glial cell line-derived neurotrophic factor (GDNF) and transplanted neural stem cells (NSCs) on stroke. Here, we explored whether transplantation of neural stem cells (NSCs) modified by *GDNF* gene provides a better therapeutic effect than native NSCs after stroke. Primary rat NSCs were transfected with GDNF plasmid (GDNF/NSCs, labeled by green fluorescent protein from AdEasy-1, GFP). Adult rats were subjected to two-hour middle cerebral artery occlusion and reperfusion, followed by infusion of NSCs (labeled with5-bromo-2′-deoxyuridine before infusion, BrdU), GDNF/NSCs and saline at 3 days after reperfusion (NSCs group, GDNF/NSCs group, control group), respectively. All rats were sacrificed at 1, 2, 3, 5, and 7 weeks after reperfusion. Modified Neurological Severity Scores (mNSS) test and H and E staining were respectively performed to evaluate neurological function and lesion volume. Immunohistochemistry was used to identify implanted cells and observe the expressions of Synaptophysin (Syp) and postsynaptic density-95 (PSD-95) and caspase-3. TdT-mediated dUTP–biotin nick-end labeling (TUNEL) was employed to observe apoptotic cells. Western blotting was used to detect brain-derived neurotrophic factor (BDNF) and NT-3 protein expression. Significant recovery of mNSS was found in GDNF/NSCs rats at 2 and 3 weeks after reperfusion compared with NSCs rats. Lesion volume in the NSCs and GDNF/NSCs groups was reduced significantly compared with control group. The number of NSCs in the GDNF/NSCs group was significantly increased in comparison with NSCs group. Moreover, Syp-immunoreactive product at 2 and 3 weeks after reperfusion and PSD-95 immunoreactive product in the GDNF/NSCs group were significantly increased compared with NSCs group. In contrast, caspase-3 positive cells and TUNEL-positive cells in the GDNF/NSCs group were significantly

* *Corresponding author*. Department of Anatomy and Neurobiology, Tongji University School of Medicine, Shanghai 200092, China. Fax: +86 21 65984636.
E-mail address: yqiongl@263.net (Q.L. Yuan).

Abbreviations: GDNF, Glial cell line-derived neurotrophic factor; NSCs, neural stem cells; GFP, green fluorescent protein; BrdU, 5′-bromo-2′-deoxyuridine; mNSS, modified Neurological Severity Scores; Syp, synaptophysin; PSD-95, postsynaptic density-95; MAP-2, microtubule-associated protein-2; GFAP, glial fibrillary acidic protein; Galc, galactocerebroside; BDNF, brain-derived neurotrophic factor; RT-PCR, reverse transcriptase-polymerase chain; MCAO, middle cerebral artery occlusion; TUNEL, TdT-mediated dUTP–biotin nick-end labeling; GDNF/NSCs, neural stem cells transfected with GDNF gene; DAB, diaminobenzidine; DMEM-F12, Dulbecco's modified Eagle's medium-F12

0006-8993/$ – see front matter
doi:10.1016/j.brainres.2009.05.100

decreased compared with NSCs group. Significant increase of BDNF protein in the GDNF/NSCs and NSCs groups was observed compared to the control group at different time points of reperfusion, and GDNF/NSCs grafting significantly increased BDNF protein expression compared to NSCs grafting. In addition, significant increase of NT-3 protein in GDNF/NSCs and NSCs groups was detected only at 1 week of reperfusion compared to control group. The results demonstrate that grafting NSCs modified by *GDNF* gene provides better neuroprotection for stroke than NSCs grafting alone.

1. Introduction

Cerebrovascular disease is a consequence of impairment of the blood supply to the brain, and ischemic strokes account for 80% of all strokes. Previous studies showed that many neurons were lost due to necrosis and apoptosis (Du et al., 1996; Charriaut-Marlangue et al., 1996), and neural functions were deficit after stroke. Up to now, there is still no effective therapeutic treatment that can improve the reconstruction of damaged cerebral tissue and its functional recovery.

Neural stem cells (NSCs) are immature cells with the ability to renew themselves and give rise to neurons, astrocytes and oligodendrocytes. Transplantation of NSCs may ameliorate the neurological deficits and recover the lost functions after stroke. Previous studies demonstrated that intravenously transplanted human NSCs could differentiate into various neural cell types and compensate for the neurological deficits following cerebral ischemia (Chu et al., 2003; Chu et al., 2004a,b; Toda et al., 2001). Moreover, NSCs could constitutively secrete neurotrophic factors in vitro (Lu et al., 2003). These studies, however, also showed that the differentiating rate of grafted NSCs into mature neurons was very low and very few of the grafted cells survived in given time. Therefore, improving the surviving rate of the grafted cells and inducing their differentiation into neurons are key points in stroke treatment with NSCs.

Neurotrophic factors have been demonstrated to possess multiple effects on brain such as neurogenesis, neurotrophic or neuroprotective actions. Neurotrophic factors also affect the survival, differentiation and proliferation significantly (Abe, 2000). Among them, GDNF has a potent neuroprotective effect on a variety of neuronal damage both in vitro and in vivo (Henderson et al., 1994; Beck et al., 1995; Li et al., 1995; Tomac et al., 1995). Topical application of GDNF decreased ischemic brain edema and number of TUNEL-positive neurons by suppressing apoptotic pathways such as caspases-1 and -3 (Kitagawa et al., 1998). The placement of GDNF-presoaked sponges in contact with the surface of cerebral cortex provided protection to the neurons within the surrounding areas (Zhang et al., 2001). Exogenous *GDNF* gene transfer reduced the infarct size in rat middle cerebral artery occlusion (MCAO) model (Hermann et al., 2001; Zhang et al., 2002) and promoted striatal neurogenesis after stroke (Kobayashi et al., 2006). However, the effects of GDNF are transient, so repeated administration into intracerebral or intraventricular space is needed. In addition, simple application of GDNF protein is difficult to administer in clinical situations because of the blood–brain barrier (Zhang et al., 2001).

In the present study, we tested whether transplantation of NSCs with overexpression of *GDNF* gene provides better neuroprotective effect than NSCs grafting alone after stroke.

2. Results

2.1. *Differentiation of NSCs in vitro and NSCs transfection by GDNF recombinant adenovirus*

Primary cultured cells survived and small aggregates of cells appeared approximately 3 days after isolation from the newborn rat cortex. After 5 days of culture, many neurospheres were seen (Fig. 1A). and these cells were nestin-positive (data not shown). After they were reseeded in the medium containing 10% calf bovine serum for 7 days, these cells were MAP2-positive, GFAP-positive and GalC-positive, respectively (Figs. 1B to D).

When NSCs were transfected by GDNF virus solution for 2 days, the NSCs showed green fluorescence in fluorescent microscopy (Fig. 1E). After these cells were further cultured in the medium with 10% calf bovine serum for 5 days, they changed into a spiny shape which indicated differentiation (Fig. 1F). In addition, RT-PCR showed that the GDNF/NSCs strongly expressed GDNF mRNA (Fig. 1G).

2.2. *Neurological assessment after NSCs and GDNF/NSCs transplantation*

No significant differences in mNSS scores were detected among the groups at 3 days after reperfusion (transplantation time point). The scores of mNSS at different times after reperfusion in the NSCs and GDNF/NSCs groups were significantly lower compared with the control group. From 1 to 7 weeks after reperfusion, the scores of mNSS in the GDNF/NSCs group were decreased compared with NSCs group, but significant decrease was observed only at 2 and 3 weeks after reperfusion. (Fig. 2A, $P<0.01$).

2.3. *Histological features and ischemic lesion volume*

H and E staining showed that the ischemic lesion regions were located to the ipsilateral frontoparietal cortex, striatum and preoptic area, where neuropil were spongy and many neurons were injured and lost after stroke. One week after reperfusion, most grafted cells in the NSCs and GDNF/NSCs groups appeared in the ipsilateral lateral ventricle, choroid plexus, subventriclar zone, and some migrated into ischemic cortex and striatum along corpus callosum and the external capsule (Figs. 3A to C). At 2 to 7 weeks after reperfusion, the grafted cells were mainly localized to the ischemic regions.

The total lesion volume in the NSCs and GDNF/NSCs groups was significantly reduced compared to the control rats. The

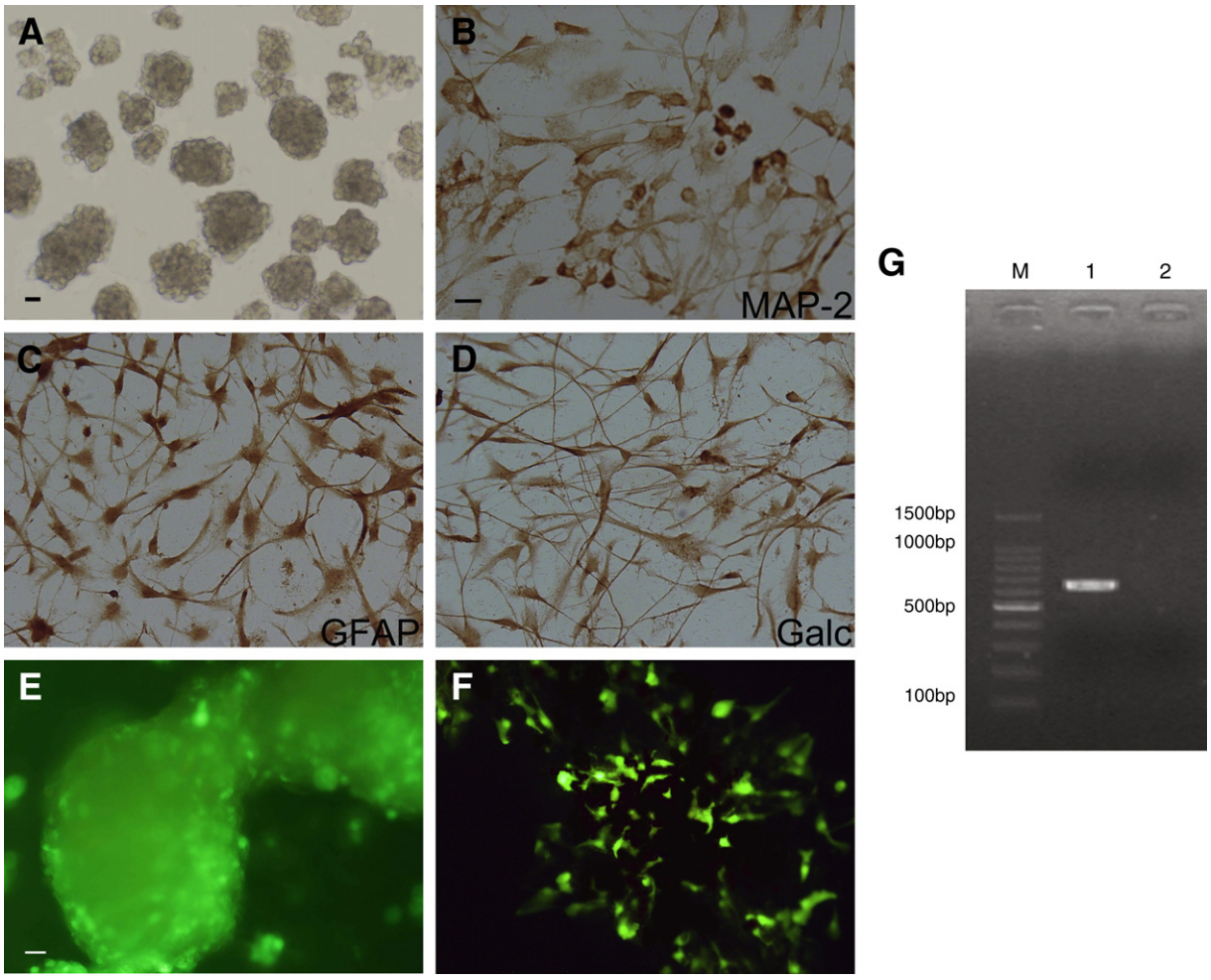

Fig. 1 – primary NSCs culture and differentiation in vitro. (A) many neurospheres derived from cerebral parenchyma of newborn rat for 5 days (phase contrast). B through D showed differentiation of NSCS in the medium containing 10% calf bovine serum: (B) MAP2-positive neurons, (C) GFAP-positive astrocytes, (D) Galc-positive oligodendrocytes. (E) NSCs/GDNF were observed in fluorescent microscope. (F) differentiation of NSCs/GDNF in vitro for 5 days (fluorescent image). (G) RT-PCR showed strong GDNF mRNA in the NSCs/GDNF (M, marker; 1 GDNF/NSCs in vitro for 5 days, 2 NSCs in vitro for 5 days). A: bar=100 μm; B through F: Bar=200 μm.

significant reduction of total lesion volume in the GDNF/NSCs rats was observed only at one week after reperfusion compared with NSCs rats (Fig. 2B, $P<0.01$).

2.4. *Distribution of NSCs after NSCs and GDNF/NSCs transplantation*

In the control group, no GFP-positive or BrdU-positive NSCs were observed. The grafted NSCs in NSCs and GDNF/NSCs groups were detected predominantly in the ischemic lesion regions, and the distributive pattern of grafted NSCs in both groups was similar. One week after reperfusion, the positive cells in both groups were predominately located in the ipsilateral ventricle, but some migrated into the ischemic frontoparietal cortex and striatum. Two weeks after reperfusion, the positive cells residing ischemic frontoparietal cortex and striatum were increased. The positive cells at 3, 5 and 7 weeks after reperfusion were significantly more than those at 1 and 2 weeks after reperfusion within each group, respectively (Figs. 3D to G).

At 1, 2, 3, 5 and 7 weeks after reperfusion, the NSCs in the ischemic regions in the GDNF/NSCs group were significantly more than those in the NSCs group (Fig. 3H, $P<0.05$).

2.5. *Upregulation of synaptic protein introduced by the transplantation*

The Syp-immunoreactive product mainly appeared in the nerve fibers at the striatum, hippocampus and hypothalamus (Figs. 4A to C). The optical density of Syp-immunoreactive product in the NSCs or GDNF/NSCs group was significantly increased compared to control group. Moreover, enhanced expression of Syp-protein in the GDNF/NSCs group at 2 and 3 weeks after reperfusion was detected compared to the NSCs group (Fig. 4M, $P<0.05$).

The PSD-95 immunoreactive product existed in the cytoplasm and process of neuron throughout the parenchyma, but

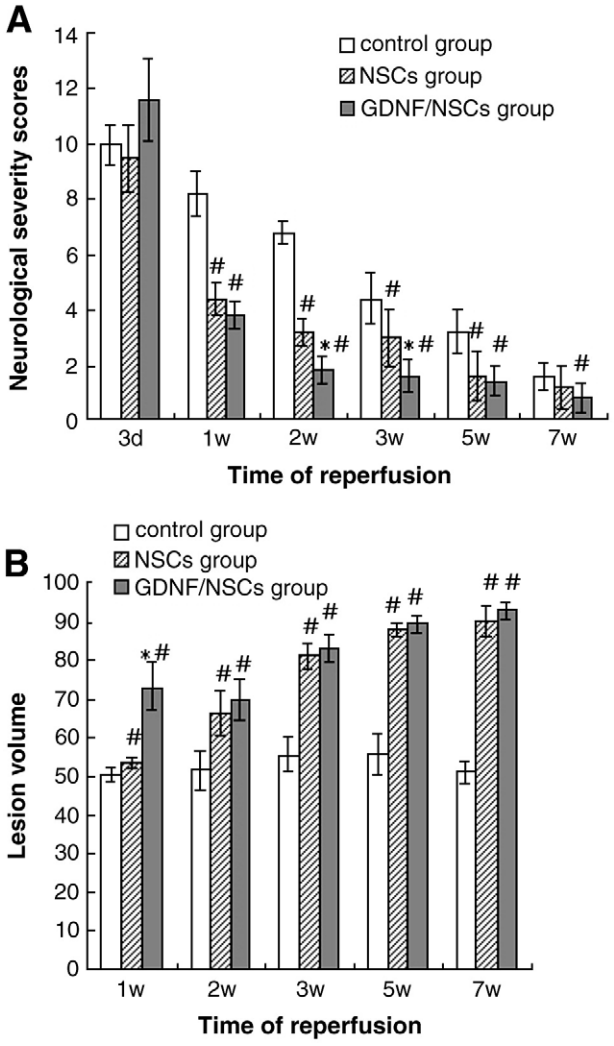

Fig. 2 – (A) Neurological functional test by mNSS after MCAO. Groups were as follows: control group, MCAO with saline infusion; NSCs group, MCAO with NSCs (5×10^5 cells); GDNF/NSCs group, MCAO with NSCs/GDNF transplantation (5×10^5 cells) at 3 days after reperfusion. $^*P<0.05$ vs NSCs group, $^\#P<0.05$ vs control group. (B) Lesion volume after MCAO in these groups. These values were calculated as: (volume of healthy side − volume of lesion side)/(volume of healthy side)×100 (%). $^\#P<0.05$ vs control group, $^*P<0.001$ vs NSCs group.

it was mainly present in the ischemic cortex, striatum and hypothalamus (Figs. 4D to F). The optical density of PSD-95 immunoreactive product in GDNF/NSCs and NSCs groups was significantly increased compared to the control group, respectively. Furthermore, the expression of PSD-95 protein in the GDNF/NSCs group was increased significantly compared to the NSCs group at given time (Fig. 4N, $P<0.05$).

2.6. *Decreased expression of caspase-3 and TUNEL-positive cells introduced by the transplantation*

Caspase-3 (Casp-3) positive cells mainly located in the ischemic hippocampus and cortex (Figs. 4G to I). The number of the positive cells of the ischemic cortex in the GDNF/NSCs and NSCs groups was significantly decreased compared to the control rats, respectively. Moreover, the number of caspase-3 positive cells in the GDNF/NSCs at 2, 3, 5 and 7 weeks after reperfusion was decreased significantly in comparison with the NSCs group (Fig. 4O, $P<0.05$).

In all rats, TUNEL-positive cells were mainly presented in ischemic striatum and frontoparietal cortex, and hippocampus in the ipsilateral hemisphere (Figs. 4 J to L). The profile of TUNEL-positive cell in all rats was similar. At one week of reperfusion, the number of TUNEL-positive cells was more, and decreased with time going. At 1 to 7 weeks of reperfusion, TUNEL-positive cells in NSCs and NSCs/GDNF grafting were significantly fewer compared to control rats. Furthermore, TUNEL-positive cells in NSCs/GDNF rats were significantly decreased in comparison with NSCs rats ((Fig. 4P, $P<0.05$).

2.7. *Increased expression of BDNF and NT-3 after transplantation*

As shown in Fig. 5, compared to the control group, grafting GDNF/NSCs and NSCs significantly increased BDNF protein expression by Western blotting analysis in rats subjected to MCAO at different time points of reperfusion. BDNF expression peaked at 3 weeks after reperfusion in both GDNF/NSCs and NSCs group. Moreover, significantly increased BDNF protein in GDNF/NSCs group was detected compared to NSCs group at different time points of reperfusion ($P<0.05$, Fig. 5). In contrast, significant increase of NT-3 protein in GDNF/NSCs and NSCs group at 1 week after reperfusion was detected compared to control group, and GDNF/NSCs significantly increased NT-3 protein expression compared to NSCs group at 1 week of reperfusion (Fig. 5, $P<0.01$). No significant increase of NT-3 protein in GDNF/NSCs and NSCs group at 2, 3, 5 and 7 weeks was observed compared to control group (Fig. 5, $P>0.05$).

3. Discussion

Our data show that transplanting NSCs modified by *GDNF* gene into rats subjected to ischemic stroke significantly improves functional outcome, and decreases lesion volume compared with control group. The number of grafted cells of host brain in the GDNF/NSCs group was significantly more than that in the NSCs group. The expression of synaptic protein in the GDNF/NSCs group was significantly increased compared with NSCs group, while the expression of caspase-3 protein and TUNEL-positive cells in the GDNF/NSCs group was significantly decreased compared with NSCs group.

Some reports showed that transplantation of NSCs may ameliorate the neurological deficits and recover the lost functions after stroke (Chu et al., 2003; Chu et al., 2004a,b; Toda et al., 2001), but Harting et al. reported that rotarod motor testing revealed significant increase in maximal speed among NSC-treated rats after traumatic brain injury compared with saline, all other motor and cognitive evaluations were not significantly different (Harting et al., 2009). Moreover, Zhu et al. reported that NSCs implantation after stroke didn't significantly improve neurological functions evaluated by mNSS (Zhu et al., 2005). In contrast, our results showed that neurological functions in the NSCs and GDNF/NSCs groups

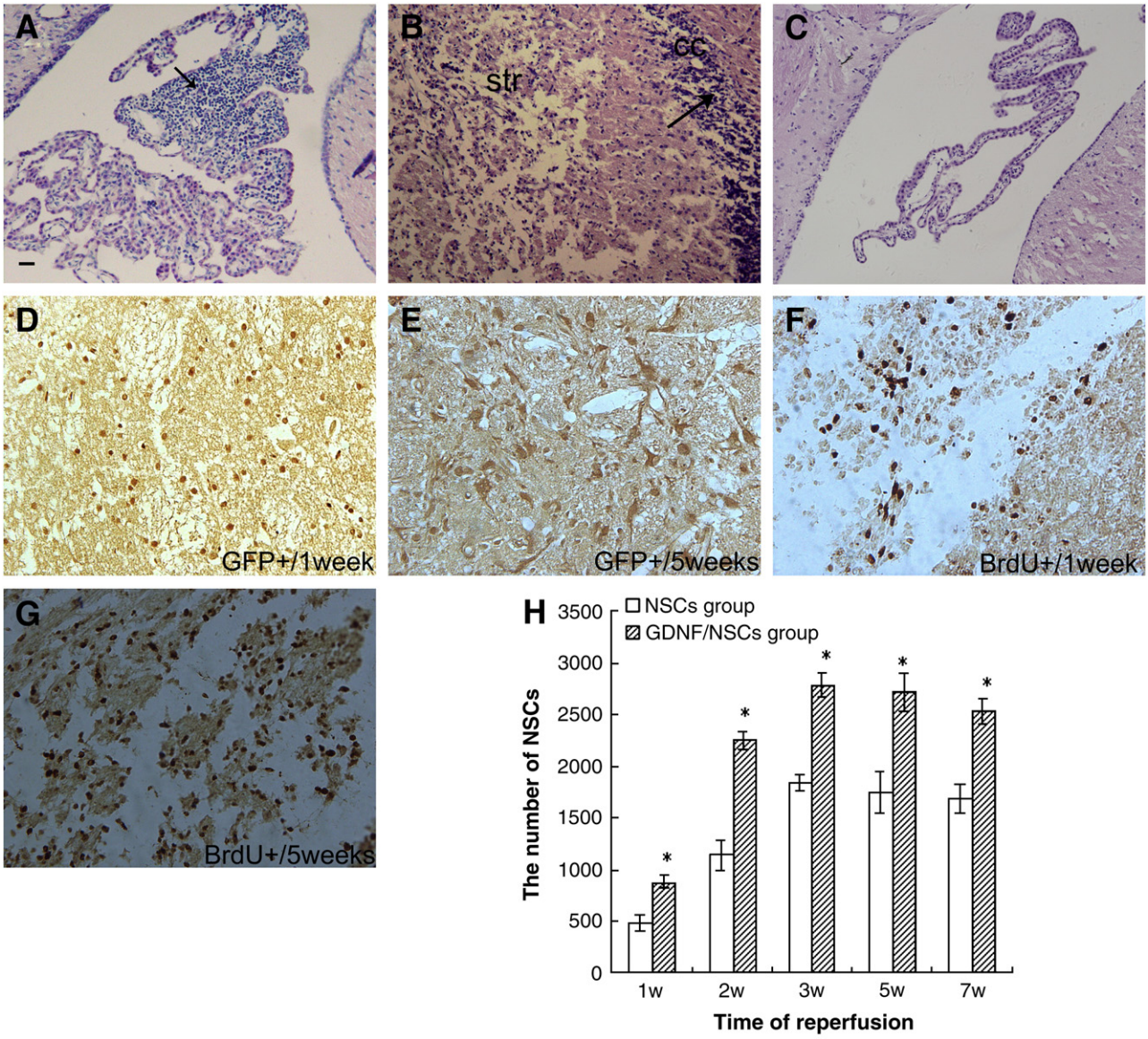

Fig. 3 – Photomicrographs show route and distribution of grafted NSCs in a recipient rat. A to B: H and E staining shows NSCs (↑) in the choroid plexus of lateral ventricle (A) and corpus callosum (B) at 1 week after reperfusion in NSCs group. C: choroid plexus of lateral ventral in control group at 1 week by H and E staining. D and E: immunohistochemical staining shows GFP-positive NSCs in the striatum of the NSCs/GDNF group at 1 and 5 weeks after reperfusion, respectively; F and G immunohistochemical staining shows BrdU-positive NSCs in the striatum of NSCs group at 1 and 5 weeks after reperfusion, respectively. The number of GFP or BrdU-positive cells in ischemic striatum and frontoparietal cortex are shown (H). *$P<0.05$ vs NSCs group. Bar=200 μm.

were significantly improved compared with control group evaluated by mNSS score from 1 to 7 weeks after reperfusion, and greater effects in the GDNF/NSCs group were observed at 2 and 3 weeks after reperfusion compared with the NSCs group.

The underlying mechanisms by which transplanted NSCs induce functional benefit after stroke are still not clear. In order to explore this mechanism, the expression of synaptic protein was detected. Our results showed that there were more synaptic protein (Syn and PSD-95) in the GDNF/NSCs or NSCs group than in the control rats ($P<0.05$), and significant increase of synaptic proteins in the GDNF/NSCs group was detected compared with NSCs group ($P<0.05$). Enhanced expression of synaptic proteins may suggest enhanced function of neurons and possible reconstruction of neural circuitry after NSCs grafting, especially GDNF/NSCs grafting. Moreover, new synaptic structures between NSCs-derived neurons and host neurons were detected at 4 weeks after reperfusion (Lu et al., 2003); in vitro NSC-derived neurons were shown to exhibit functional synaptic formation by them in approximately 2–4 weeks after differentiation (Song et al., 2002). Taken together, reconstruction of neural circuitry may contribute to part of the functional recover after stroke.

Up to now, a continuous observation of lesion volume has not been reported after NSCs grafting. Ishibashi et al. reported that the total infarction volume in the NSC-grafted animals was reduced significantly compared with control group at

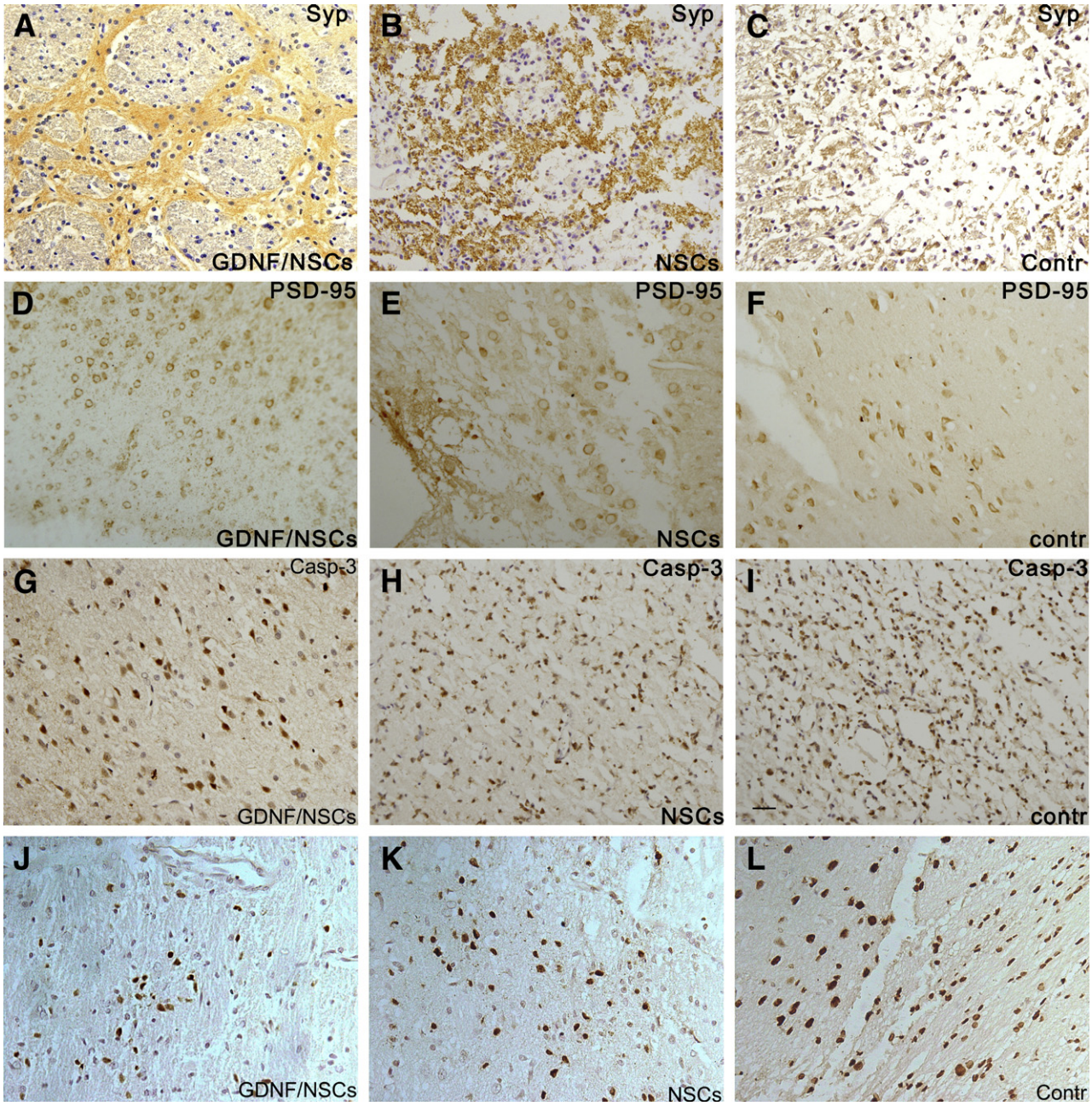

Fig. 4 – A through C show expression of synaptophysin (Syp) in the striatum of the GDNF/NSCs, NSCs and control groups at 2 weeks after reperfusion, respectively; D through F show expression of PSD-95 in the frontoparietal cortex of the GDNF/NSCs, NSCs and control groups at 5 weeks after reperfusion; G through I show caspase-3 (Casp-3) expression in the frontoparietal cortex of the GDNF/NSCs, NSCs and control groups at 3 weeks after reperfusion, respectively. J through L show TUNEL-positive cells in frontoparietal cortex of the GDNF/NSCs (J), NSCs (K) and control groups (L) at 2 weeks after reperfusion. The optical density of synaptophysin (M) and PSD-95 (N) and the number of caspase-3 positive cells (O) and TUNEL (P) are shown. $^{*}P<0.05$ vs NSCs, $^{\#}P<0.05$ vs control group. Bar=200 μm.

4 weeks after transplantation ($P<0.05$) (Ishibashi et al., 2004). Chu et al. reported that the infarct size in the NSCs grafting was not statistically different at 7 days after stroke compared with control group (Chu et al., 2004a,b), but it was significantly decreased compared with ischemia group at 5 weeks in his another paper (Chu et al., 2005). In our results, significant reduction of lesion volume in the GDNF/NSCs and NSCs group was detected compared with control group. Although the lesion volume in the GDNF/NSCs group at different times after reperfusion was reduced compared to the NSCs group, no significant reduction in the GDNF/NSCs group was detected compared with NSCs group except 1 week after reperfusion (4 days after transplantation) ($P<0.01$). Zhang et al. reported that treatment with Ad–GDNF significantly reduced the infarct volume when immediately administered after the reperfusion, but became insignificant when administered at 1 h after

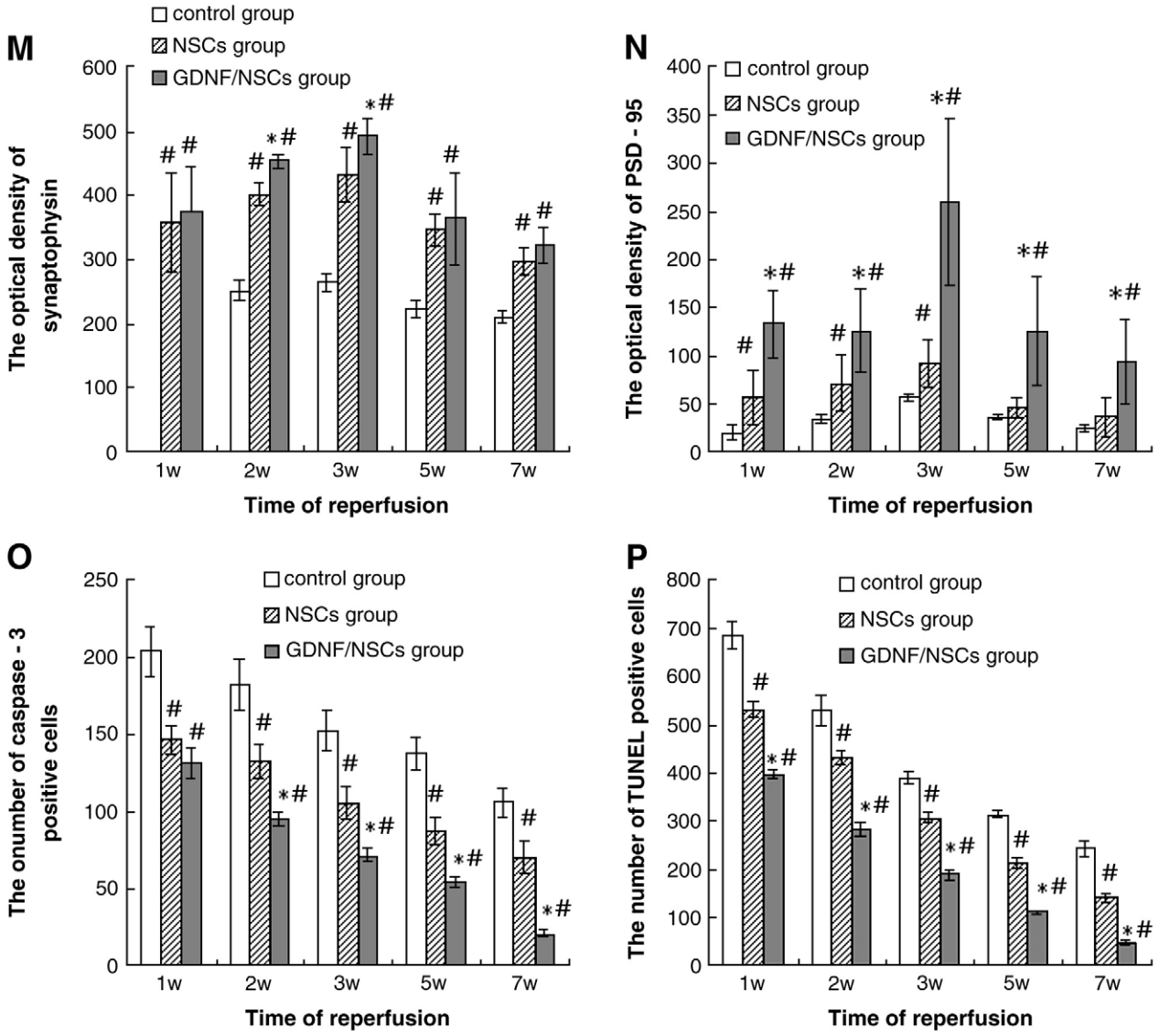

Fig. 4 (*continued*).

the reperfusion as were the cases treated with vehicle-groups (Zhang et al., 2002). The pathological mechanisms of transient ischemia are complicated, including acute necrosis and delayed apoptosis (Du et al., 1996; Charriaut-Marlangue et al., 1996). Acute necrosis is mainly due to rapid blockade of regional blood flow. In contrast, delayed apoptotic process is theoretically controlled. When GDNF/NSCs or NSCs were transplanted in the present study at 3 days after reperfusion, delayed apoptosis was interfered. Exogenous GDNF and NTs from host brain may prevent neuronal apoptosis (Harting et al., 2009; Yuan et al., 2001) and reduce the injury volume. It was possible that GDNF protein derived from grafted NSCs peaked at 4 days after reperfusion, which significantly reduced the number of apoptotic cells, therefore reduced the injury volume. In addition, present results showed that caspase-3 protein and TUNEL-positive cells in the GDNF/NSCs group was decreased significantly compared with NSCs group, which strongly suggested that GDNF may protect neuron by way of inhibiting apoptosis.

In addition, our results showed the number of grafted cells in the GDNF/NSCs rats was more than that in the NSCs rats. Moreover, the grafted NSCs in the GDNF/NSCs appeared some neurites from 3 to 7 weeks after reperfusion. These strongly suggested that grafted NSCs modified by *GDNF* gene exhibited higher surviving rate and differentiation of grafted NSCs in host brain tissue after stroke, which are very crucial for improving NSCs therapeutic effect for stroke. The underlying mechanism may be due to the fact that GDNF/NSCs grafting increased protein expression of BDNF and NT-3, which is crucial for neuron survival. The recovery of neurological behavior and pathological changes of injured tissue may partly result from more survival of grafted NSCs.

In conclusion, we have demonstrated that grafting NSCs modified by *GDNF* gene significantly improves functional recovery, promotes survival of grafted cells, and enhances expression of synaptic protein, BDNF and NT-3 and decreases caspase-3 protein compared with NSCs grafting

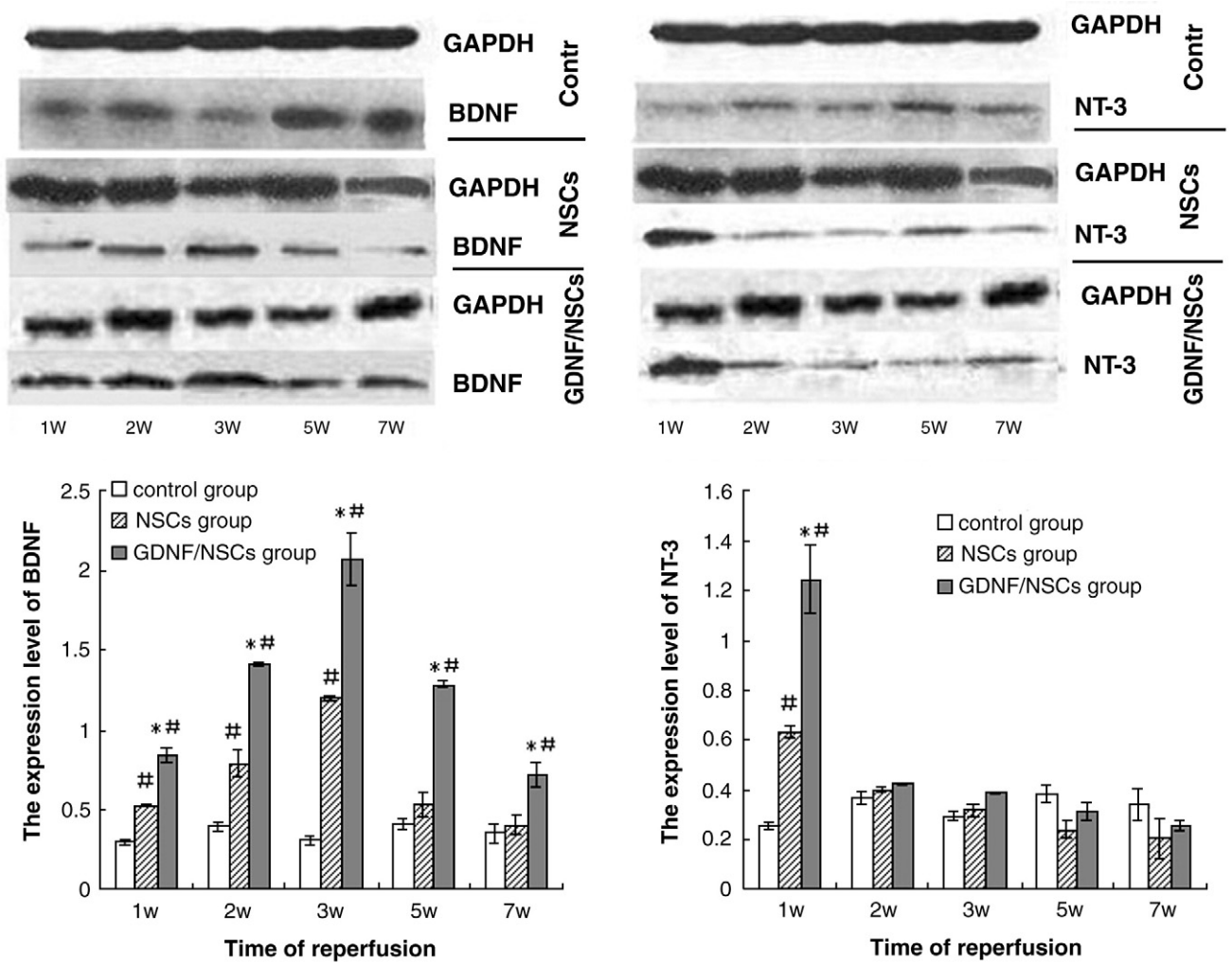

Fig. 5 – Western blotting analyzing BDNF and NT-3 protein expression in the ipsilateral hemisphere in rats subjected to MCAO and following GDNF/NSCs and NSCs transplantation. GAPDH was the internal control. Each value represents mean ± SD ($n=3$). $^{\#}P<0.01$ vs control group; $^{*}P<0.01$ vs NSCs group.

alone. These results suggest that GDNF/NSCs grafting provides better neuroprotection for stroke than NSCs grafting alone.

4. Experimental procedures

4.1. *Animals*

Adult male or female Wistar rats weighing 250–300 g were used in all experiments (inbred strain, Animal House Center, third Military Medical University, Zhongqing, PR China). Animals were housed in a colony room under controlled temperature, humidity, and a 12-h light/dark cycle, with food and water available throughout animal experiments. Animal care and experimental protocols were approved by the Chinese Academy of Sciences, PR China, ensuring that animal numbers and suffering were kept to the minimum. All the experiments were conformed to the international guidelines on the ethical use of animals.

4.2. *pAdEasy-1-pAdTrack CMV vector expressing rat GDNF gene*

We prepared a pAdEasy-1-pAdTrack CMV vector expressing the rat *GDNF* gene (pAdEasy-1-pAdTrack CMV-GDNF) (Yan et al., 2005). Adeasy-1 plasmid contains gene of green fluorescent protein (GFP). To determine viral concentration, the viral solution was incubated in 0.1% sodium dodecyl sulphate and A260 was measured. The viral titres of pAdEasy-1-pAdTrack CMV-GDNF was 1×10^{9} PFU/mL. The GDNF virus solution was kept at −80 °C for NSCs transfection.

4.3. *Isolation and differentiation of NSCs in vitro*

Primary NSCs were isolated from cerebral cortex of newborn Wistar rats as described previously (Yin et al., 2005). Many neurospheres were observed at 7 days of culture. After passaging two generations, neurospheres were dissociated into single cells by incubation in 0.1% trypsin-ethylenediamine tetra-acetic acid at 37 °C for 2 min and then centrifugation in

10 ml of Dulbecco's modified Eagle's medium-F12 (DMEM-F12) medium containing 4% bovine serum albumin at 110 ×*g* for 5 min. For immunocytochemistry, neurospheres from single cells were grown on coverslips with serum-free medium for 3–7 days and processed for immunocytochemistry. Cultures were fixed by 4% paraformaldehyde for 30 min. The cells were incubated with blocking solution (2% bovine serum albumin and 0.1% Triton X-100 in 0.1 mol/L of PBS, pH 7.6) for 20 min in a moisture chamber to block nonspecific binding. The cells were then incubated with monoclonal nestin antibody (a marker for NSCs, 1:100 dilution, DAKO, Denmark) overnight at 4 °C. The next day, cells were washed in PBS three times for 5 min each time and then incubated with a secondary antibody peroxidase-conjugated rabbit anti-mouse IgG (1:100 dilution, Santa Cruz Bioctechnology, USA) at 37 °C for 2 h. After washing three times in PBS, cells were incubated with avidin–biotin complex (1:100 dilution, Santa Cruz Bioctechnology, USA) at 37 °C for 1 and a half hours, Diaminobenzidine (DAB, Sigma, USA) was then used as a chromogen for light microscopy. A negative control was carried out using the same procedures without primary antibody.

To observe the differentiation of NSCs in vitro, the neurospheres were transferred to coverslips coated with poly-L-lysine (10 μg/mL, Sigma) and cultured in DMEM-F12. After 2 days of culture, NSCs were differentiated using DMEM-F12 with 10% fetal bovine serum medium. Cells were then fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) and labeled with cell-specific markers for neurons (microtubule-associated protein 2, MAP-2, 1:200 dilution, Boehringer Mannheim), astrocytes (glial fibrillary acidic protein, GFAP, 1:200 dilution, DAKO, Denmark), and oligodendrocytes (galactocerebroside, Galc, 1:100 dilution, DAKO, Denmark). Then the similar procedures as above were performed. A negative control was carried out using the same procedures without primary antibody.

4.4. *Transfection of NSCs with GDNF recombinant virus solution*

The neurospheres of NSCs were gently triturated into single-cell suspension at a density of 1.25×10^6cell/mL. 200 μL single-cell suspensions were seeded in each well of a 24-well plate. 5 μL GDNF virus solution was then added to each well and was incubated for 2 h at 37 °C in a 5% CO_2 incubator. After adding 300 μL culture medium in each well, the mixed medium was further incubated for 48 h. The cell suspension was then centrifuged, and the GDNF-transfected NSCs (GDNF/NSCs) were harvested for grafting. The expression of GDNF in NSCs was examined by a reverse transcriptase-polymerase chain reaction (RT-PCR) assay 4 days after transfection.

4.5. *Stroke treatment with transplantation of GDNF/NSCs and NSCs*

4.5.1. *Animal transient middle cerebral artery occlusion model*

Adult male Wistar rats ($n=75$, weighing 250 to 300 g) were used in our experiment. Briefly, rats were anesthetized with 1% pentobarbital sodium in 0.9%NaCI (30 mg/kg, i.p.). Rectal temperature was controlled at 37 °C with a feedback-regulated water heating system. Transient (2 h) middle cerebral artery occlusion (MCAO) was induced by using a previously described method of intraluminal vascular occlusion (Yuan et al., 2007).

4.5.2. *Implantation procedures and experimental groups*

Single-cell suspension was prepared at a density of 2.5×10^7 cells/mL for transplantation. For labeling in vivo in the NSCs group, NSCs were pretreated with 10 μM of 5-bromo-2′-deoxyuridine (BrdU) for 3 days before grafting.

At 3 days after reperfusion, the animals were reanesthetized with pentobarbital sodium and then placed in a stereotaxic frame (Angle Two™ Stereotaxic Instrument w/Rat Atlas Product: #464601, USA). An incision was made to expose bregma. Burr holes were drilled for ipsilateral lateral ventricle (AP=−0.8 to 1.0 mm, L=−1.8 to 2.0 mm, V=−4.0 to 5.0 mm). 5×10^5 cells (NSCs, GDNF/NSCs) in the total 20 μL fluid were transplanted into ipsilateral lateral ventricle at a speed of 5 μL/min. The control group underwent the same injection with 20 μL saline. Immunosuppressants or antibiotics were not used in all animals.

4.5.3. *Neurological functional tests*

Pre-MCAO and Post-MCAO, all rats were housed in a colony room under controlled temperature, humidity, and a 12-h light/dark cycle, with food and water available, A modified

Table 1 – Modified Neurological Severity Score points.

Motor tests	
Raising rat by tail	3
Flexion of forelimb	1
Flexion of hindlimb	1
Head moved >10° to vertical axis within 30 s	1
Placing rat on floor (normal=0; maximum=3)	3
Normal walk	0
Inability to walk straight	1
Circling toward paretic side	2
Falls down to paretic side	3
Sensory tests	2
Placing test (visual and tactile test)	1
Proprioceptive test (deep sensation, pushing paw against table edge to stimulate limb muscles)	1
Beam balance tests (normal=0; maximum=6)	6
Balances with steady posture	0
Grasps side of beam	1
Hugs beam and 1 limb falls down from beam	2
Hugs beam and 2 limbs fall down from beam, or spins on beam (>60 s)	3
Attempts to balance on beam but falls off (>20 s)	5
Falls off; no attempt to balance or hang on to beam (<20 s)	6
Reflex absence and abnormal movements	4
Pinna reflex (head shake when auditory meatus is touched)	1
Corneal reflex (eye blink when cornea is lightly touched with cotton)	1
Startle reflex (motor response to a brief noise from snapping a clipboard paper)	1
Seizures, myoclonus, myodystony	1
Maximum points	18

One point is awarded for inability to perform the tasks or for lack of a tested reflex: 13–18, severe injury; 7–12, moderate injury; 1–6, mild injury.

Neurological Severity Score (mNSS) test was performed at 3 days (implantation time point) and 1, 2, 3, 5, 7 weeks after reperfusion by an investigator who was blinded to the experimental groups as described previously (Chen et al., 2001). The mNSS is graded on a scale of 0 to18 (normal score, 0; maximal deficit score,18). In the severity scores of injury, 1 score point is awarded for the inability to perform the test or for the lack of a tested reflex; thus, the higher the score, the more severe is the injury (seen in Table 1).

4.5.4. *Histological and immunohistochemical assessment*

At the end of the given time, animals were anesthetized deeply with pentobarbital sodium, sacrificed, and fixed by perfusion with 4% paraformaldehyde. The brain was sectioned into 7 equally spaced (2 mm) coronal blocks and was embedded in paraffin. The 6-μm coronal sections were stained with hematoxylin/eosin (H and E) for calculation of the ischemic lesion areas, which were traced using NIH Image software (Image-Pro PlusTM, Media Cybernetics), and the total lesion volume (indirect lesion volume) of the ipsilateral hemisphere was calculated as a percentage of the volume of the contralateral hemisphere, as reported previously (Swanson et al., 1990).

Immunohistochemical staining was used to identify grafted cells in vivo and assess the expression of synaptic protein and apoptotic protein. Briefly, a series of 6 μm-thick sections at various levels (for bregma −0.2 to −1.6 mm) were selected. After deparaffinization, sections were placed in boiled citrate buffer (pH 6.0) within a microwave oven (650 to 720 W). After being blocked in normal 5% horse serum at 37 °C for 30 min, Sections were treated with primary antibodies: the monoclonal antibody against Brdu (1:100 dilution; DAKO) or polyclonal rabbit antibody against GFP (1:100 dilution, DAKO), monoclonal antibody against synaptophysin (Syp, a marker of presynaptic protein, 1:100 dilution, Wuhan Boster Biological Technology, China), postsynaptic synaptic protein-95 (PSD-95, for a marker of postsynaptic elements, 1:100 dilution, Wuhan Boster Biological Technology, China) and caspase-3 (a marker of apoptosis, 1:100 dilution, Santa Cruz Bioctechnology, USA). After sequential incubation with biotinylated IgG, the sections were treated with an HRP-streptavidin (SP kit, Wuhan Boster Biological Technology, China), DAB was then used as a chromogen for light microscopy. Counterstaining of the sections by hematoxylin was also performed. All incubations were performed in a humidified chamber. Negative control sections from each animal were identically stained, except that the primary or the secondary antibody was omitted.

4.6. *In situ detection of DNA fragmentation (TdT-mediated dUTP–biotin nick-end labeling, TUNEL)*

TUNEL was employed to identify the number of cell exhibiting DNA fragmentation by means of light microscope as previously described (Gavrieli et al., 1992). Briefly, after deparaffinizing, the protein in brain sections was digested using the proteinase K and quenching endogenous peroxidase activity with 2% H_2O_2 in PBS. The slides were placed in the equilibration buffer and then in working-strength TdT enzyme, followed by working-strength stop/wash buffer. After 2 drops of anti-digoxigenin-peroxidase were applied to the slides, peroxidase was detected with DAB. Negative controls were performed using the distilled water for TdT enzyme in the preparation of working-strength. Sections were counterstained with hematoxylin.

4.7. *Western blotting analysis for BDNF and NT-3 protein*

Rats in each experimental group ($n=3$) were decapitated, and forebrain tissue was immediately obtained. Each specimen was dissected on a bed of ice into hemisphere ipsilateral to the MCAO. The segments were quick frozen in isopentane and stored at −80 °C until homogenization. Then, these segments were thawed on ice, and wet weight in grams was rapidly measured. The tissue pieces were homogenized by adding a 1:5 tissue weight to protein extraction buffer, containing 0.9% NaCI, 7 mM β-mercaptoethanol, 50 mM Tris–HCl (pH 7.5), 2 mM EDTA, and 1% sodium dodecyl sulphate (SDS), in a glass homogenizer. The protein concentration of samples was determined using a BCA protein assay kit (Beyotime Institute of Biotechnology, China). Homogenate samples (50 μg) were mixed with an equal volume of a sample buffer and heated at 95 °C for 5 min and resolved by SDS-7.5% polyacrylamide gel electrophoresis (PAGE) and transferred onto nitrocellulose membranes. Blots were blocked at room temperature for 1 h in a blocking buffer, Tris-buffered saline-Tween (TBS-T) containing 5% dried milk. Blots were incubated in the primary antibody, mouse anti-rat BDNF (A and D, USA, dilution 1:500), NT-3 (A and D, USA, dilution: 1:500) and GAPDH (dilution: 1:1000), diluted in the blocking buffer for 1 h at room temperature and were washed extensively with TBS-T. They were then incubated in a horseradish peroxidase-conjugated secondary antibody, diluted in the blocking buffer (dilution: 1:1000) for 1 h and washed for an additional hour. The protein band of interest was visualized using an ECL chemiluminescence system (ECL plus; Amersham Biosciences, NJ, USA) and the density of each band was quantified by using an image analysis software. GAPDH was the internal control.

4.8. *Image analysis and statistical analysis*

The number and optical density of immunoreactive product in the coronal section from bregma −0.2 to −1.4 mm were counted in 3 to 4 sections per animal ($n=5$). The number of positive cells and optical density for immunohistochemical staining were measured on each section using a computer-based C-CCD camera system (Olympus BX 40; Olympus optical Co., Tokyo, Japan Olympus, DP70). Syp and caspase-3 were measured in the striatum and hippocampus of ipsilateral cerebral tissue under a 40× objective lens. PSD-95, TUNEL-positive cells and grafted NSCs were measured in the striatum and frontoparietal cortex of ipsilateral cerebral tissue under a 40× objective lens.

All data study are presented as means ± SD. Differences between mean values for different times within a group were analyzed using one-way ANOVA, followed by post hoc tests. And independent sample *t*-test was performed to detect the difference between mean values for the same time points among these groups. Statistical significance was set at $P<0.05$.

REFERENCES

Abe, K., 2000. Therapeutic potential of neurotrophic factors and neural stem cells against ischemic brain injury. J. Cereb. Blood. Flow. Metab. 20, 1393–1408.

Beck, K.D., Valverde, J., Alexi, T., Poulsen, K., Moffat, B., Vandlen, R.A., Rosenthal, A., Hefti, F., 1995. Mesencephalic dopaminergic neurons protected by GDNF from axotomy-induced degeneration in the adult brain. Nature 373, 339–341.

Charriaut-Marlangue, C., Margaill, I., Represa, A., Popovici, T., Plotkine, M., Ben-Ari, Y., 1996. Apoptosis and necrosis after reversible focal ischemia: an in situ DNA fragmentation analysis. J. Cereb. Blood. Flow. Metab. 16, 186–194.

Chen, J., Sanberg, P.R., Li, Y., Wang, L., Lu, M., Willing, A.E., Sanchez-Ramos, J., Chopp, M., 2001. Intravenous administration of human umbilical cord blood reduces behavioral deficits after stroke in rats. Stroke 32, 2682–2688.

Chu, K., Kim, M., Jeong, S.W., Kim, S.U., Yoon, B.W., 2003. Human neural stem cells can migrate, differentiate, and integrate after intravenous transplantation in adult rats with transient forebrain ischemia. Neurosci. Lett. 343, 129–133.

Chu, K., Kim, M., Park, K.I., Jeong, S.W., Park, H.K., Jung, K.H., Lee, S.T., Kang, L., Lee, K., Park, D.K., Kim, S.U., Roh, J.K., 2004a. Human neural stem cells improve sensorimotor deficits in the adult rat brain with experimental focal ischemia. Brain. Res. 1016, 145–153.

Chu, K., Kim, M., Chae, S.H., Jeong, S.W., Kang, K.S., Jung, K.H., Kim, J., Kim, Y.J., Kang, L., Kim, S.U., Yoon, B.W., 2004b. Distribution and in situ proliferation patterns of intravenously injected immortalized human neural stem-like cells in rats with focal cerebral ischemia. Neurosci. Res. 50, 459–465.

Chu, K., Park, K., Lee, S.T., Jung, K.H., Ko, S.Y., Kang, L., Sinn, D.I., Lee, Y.S., Seung, U., Kim, S.U., Kim, M., Roh, J.K., 2005. Combined treatment of vascular endothelial growth factor and human neural stem cells in experimental focal cerebral ischemia. Neurosci. Res. 53, 384–390.

Du, C., Hu, R., Csernansky, C.A., Hsu, C.Y., Choi, D.W., 1996. Very delayed infarction after mild focal cerebral ischemia: a role for apoptosis. J. Cereb. Blood Flow Metab. 16, 195–201.

Gavrieli, Y., Sherman, Y., Ben-Sasson, S.A.H., 1992. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J. Cell Biol. 119, 493–501.

Harting, M.T., Sloan, L.E., Jimenez, F., Baumgartner, J., Cox, C.S., 2009. Subacute neural stem cell therapy for traumatic brain injury. J. Surg. Res. 153, 188–194.

Henderson, C.E., Phillips, H.S., Pollock, R.A., Davies, A.M., Lemeulle, C., Armanini, M., Simpson, L.C., Moffet, B., Vandlen, R.A., Koliatsos, V.E., Rosenthal, A., 1994. GDNF: a potent survival factor for motoneurons present in peripheral nerve and muscle. Science 266, 1062–1064.

Hermann, D.M., Kilic, E., Kugler, S., Isenmann, S., Bahr, M., 2001. Adenovirus-mediated GDNF and CNTF pretreatment protects against striatal injury following transient middle cerebral artery occlusion in mice. Neurobiol. Dis. 8, 655–666.

Ishibashi, S., Sakaguchi, M., Kuroiwa, T., Yamasaki, M., Kanemura, Y., Shizuko, I., Shimazaki, T., Onodera, M., Okano, H., Mizusawa, H., 2004. Human neural stem/progenitor cells, expanded in long-term neurosphere culture, promote functional recovery after focal ischemia in Mongolian gerbils J. Neurosci. Res. 78, 215–223.

Kitagawa, H., Hayashi, T., Mitsumoto, Y., Koga, N., Itoyama, Y., Abe, K., 1998. Reduction of ischemic brain injury by topical application of glial cell line-derived neurotrophic factor after permanent middle cerebral artery occlusion in rats Stroke 29, 1417–1422.

Kobayashi, T., Ahlenius, H., Thored, P., Kobayashi, R., Kokaia, Z., Lindvall, O., 2006. Intracerebral infusion of glial cell line-derived neurotrophic factor promotes striatal neurogenesis after stroke in adult rats. Stroke. 37, 2361–2367.

Li, L., Wu, W., Lin, L.F., Lei, M., Oppenheim, R.W., Houenou, L.J., 1995. Rescue of adult mouse motoneurons from injury-induced cell death by glial cell line-derived neurotrophic factor. Proc. Natl. Acad. Sci. U. S. A. 92, 9771–9775.

Lu, P., Jones, L.L., Snyder, E.Y., Tuszynski, M.H, 2003. Neural stem cells constitutively secrete neurotrophic factors and promote extensive host axonal growth after spinal cord injury. Exp. Neurol. 181, 115–129.

Song, H.J., Stevens, C.F., Gage, F.H., 2002. Neural stem cells from adult hippocampus develop essential properties of functional CNS neurons. Nat. Neurosci. 5, 438–445.

Swanson, R.A., Morton, M.T., Tsao-Wu, G., Savalos, R.A., Davidson, C., Sharp, F.R., 1990. A semiautomated method for measuring brain infarct volume. J. Cereb. Blood Flow Metab. 10, 290–293.

Toda, H., Takahashi, J., Iwakami, N., Kimura, T., Hoki, S., Mozumi-Kitamura, K., Ono, S., Hashimoto, N., 2001. Grafting neural stem cells improved the impaired spatial recognition in ischemic stroke. Neurosci. Lett. 316, 9–12.

Tomac, A., Lindqvist, E., Lin, L.F., Ogren, S.O., Young, D., Hoffer, B.J., Olson, L., 1995. Protection and repair of the nigrostriatal dopaminergic system by GDNF in vivo. Nature 373, 335–339.

Yan, N.H., Chen, Q.Y., Cao, G.Q., Lu, Y.L., Zhang, F.Q., Yaun, Q.L., Xio, Q.I., 2005. Construction of recombinant adeno-associated virus vector expressing gliai cel line-derived neurotrophic factor labeled by green fluorescent protein. Chin. J. Clin. Rehabil. 9, 26–28.

Yin, G.C., Luan, Z., Qu, S.G., Guo, A.L., 2005. Culture of neural stem cells from cerebral cortex of newborn rats and its effect in cell replace in siblings. Chin. J. Perinat. Med. 8, 255–258.

Yuan, Q.L., Lan, S.Q., Li, R.X., Yang, H.J., Zhang, G.P., 2001. The expressive characteristics of neuron of immediately early gene and neurotrophic factor TGF-β, NGF, GDNF following ischemia-reperfusion in rat brain. Chin. J. Neuroanat. 17, 77–81.

Yuan, Q.L., Yang, C.X., Xu, P., Gao, X.Q., Deng, L., Chen, P., Sun, Z.L., Chen, Q.Y., 2007. Neuroprotective effects of ginsenoside Rb1 on transient cerebral ischemia in rats. Brain Res. 1167, 1–12.

Zhang, W.R., Hayashi, T., Iwai, M., Nagano, I., Sato, K., Manabe, Y., Abe, K., 2001. Time dependent amelioration against ischemic brain damage by glial cell line-derived neurotrophic factor after transient middle cerebral artery occlusion in rat. Brain Res. 903, 253–256.

Zhang, W.R., Sato, K., Iwai, M., Nagano, I., Manabe, Y., Abe, K., 2002. Therapeutic time window of adenovirus-mediated GDNF gene transfer after transient middle cerebral artery occlusion in rat. Brain Res. 947, 140–145.

Zhu, W., Mao, Y., Zhao, Y., Zhou, L.F., Wang, Y., Zhu, J.H., Zhu, Y., Yang, G.Y., 2005. Transplantation of vascular endothelial growth factor-transfected neural stem cells into the rat brain provides neuroprotection after transient focal cerebral ischemia. Neurosurgery 57, 325–333.