

# Viability-Dependent Promoting Action of Adult Neural Precursors in Spinal Cord Injury

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The aim of the study was the assessment of the effects of adult neural stem cell (NSC) transplantation in a mouse model of spinal cord injury (SCI). The contusion injury was performed by means of the Infinite Horizon Device to allow the generation of reproducible traumatic lesion to the cord. We administered green fluorescent-labeled (GFP-)NSCs either by intravenous (i.v.) injection or by direct transplantation into the spinal cord (intraspinal route). We report that NSCs significantly improved recovery of hind limb function and greatly attenuated secondary degeneration. The i.v. route of NSC administration yielded better recovery than the intraspinal route of administration. About 2% of total i.v.-administered NSCs homed to the spinal cord injury site, and survived almost undifferentiated; thus the positive effect of NSC treatment cannot be ascribed to damaged tissue substitution. The NSCs homing to the injury site triggered, within 48 h, a large increase of the expression of neurotrophic factors and chemokines. One wk after transplantation, exogenous GFP-NSCs still retained their proliferation potential and produced neurospheres when recovered from the lesion site and cultured *in vitro*. At a later time, GFP-NSC were phagocytated by macrophages. We suggest that the process of triggering the recovery of function might be strongly related to the viability of GFP-NSC, still capable *ex vivo* of producing neurospheres, and their ability to modify the lesion environment in a positive fashion.

Online address: <http://www.molmed.org>

doi: 10.2119/2008-00077.Bottai

## INTRODUCTION

Primary injury is an immediate response resulting from the direct action of mechanical force on the spinal cord. Secondary injury consists of all pathological events initiated by the original mechanical insult. The secondary injury following SCI is fundamentally the result of a stroke-like ischemic insult; the biochemical and metabolic events associated to the vascular dysregulation are numerous, and lead to the progressive degeneration that may exceed the early mechanical damage. A complicated interplay of pro-inflammatory molecules, anti-inflammatory molecules, and migratory cells also mediates the pathophysiology of the spinal cord following

injury. Within the first 12 h, neutrophils first appear, with lymphocytes and microglia then mobilized to the injury site within 24–48 h. Among pro-inflammatory cytokines, much interest has been focused on MIP-2, TNF $\alpha$ , IL-1 $\beta$ , and IL-6, which are key mediators of inflammation following CNS lesions. Overproduction of MIP-2 into an injured spinal cord was associated with neutrophil infiltration, indicating a key role played by this mediator in inflammatory cell recruitment induced by SCI (1,2). In keeping with this suggestion, reparixin (a blocker of IL-8 receptor) treatment completely prevented neutrophil and ED-1-positive cell infiltration into the injured spinal cord (3). The complex summation of pri-

mary and secondary degeneration events determines the extent of tissue damage that normally extends much farther than the limits of the mechanical impact. The attenuation of cellular and molecular abnormalities underlying the secondary injury may result, then, in a dramatic improvement in recovery from SCI-derived disability.

Since the loss of CNS neurons may not be replaced by the proliferation of the surviving ones, intraspinal transplantation of exogenous neuronal cells or tissue has been accepted for a long time as a way to obtain a partial reconstruction of the lost cord tissue and to promote recovery of neurological function (4). The transplantation of fetal cerebral tissue has been pursued for a long time. Recently, an alternative to the use of fetal human tissue for therapeutic transplantation has arisen due to the discovery that both embryonic and adult mammalian CNS contains multi-potent stem cells that, although expandable as undifferentiated elements, can be triggered to differentiate into neurons, astro- and

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oligodendroglia, and could be used for neural engraftment (5,6).

Adult stem cells have been isolated from numerous adult tissues and other non-embryonic sources, and have demonstrated a surprising ability for transformation into other tissue or cell types and for repair of damaged tissues (7–10).

Regeneration or replacement of dead or damaged cells is the primary goal of regenerative medicine and one of the prime motivations for the study of stem cells. NSCs can participate in repair of damage and may be useful in the treatment of degenerative brain conditions (11). In models of Parkinson's disease, NSCs have been observed to integrate and survive for extended periods of time (12), and to rescue and prevent the degeneration of endogenous dopaminergic neurons (13).

Grafted embryonic stem cells, marrow stromal cells, and fetal NSCs survive and improve functional recovery after SCI (14–16). Here, it is shown that adult NSCs applied via intraspinal or i.v. injection within 18 h after SCI also counteract secondary degeneration and promote the recovery of function after acute SCI, as reported previously for acutely administered erythropoietin (17,18).

Exogenous NSCs could be recovered live and regrown *in vitro* up to 7 d after administration; in addition, NSCs modified the lesion environment by promoting the expression of neurotrophic factors, and also produced the main enhancement of the rate of recovery during this initial period. Later, NSCs were phagocytosed by macrophages, but the extent of functional recovery was maintained.

## MATERIALS AND METHODS

### Animal Care

Adult CD1 male mice 28–30 g in weight and all the procedures were taken with the approval of the Review Committee of the University of Milan and met the Italian Guidelines for Laboratory Animals which conform to the European Communities Directive of November 1986 (86/609/EEC).

The animals were kept for at least 3 d before the experiments in standard conditions ( $22 \pm 2^\circ \text{C}$ , 65% humidity, and artificial light between 08:00 a.m. to 08:00 p.m.).

### NSCs Preparation and Validation

Primary cultures and cultivation of the NSCs from adult mice, their differentiation, and their immunostaining were performed as described elsewhere (19); briefly, 2- to 3-month-old CD-1 albino mice carrying the transgenic gene for green fluorescent protein (GFP) under the control of the  $\beta$ -actin promoter (20) were anesthetized by intraperitoneal (i.p.) injection of 4% chloral hydrate (0.1 mL/10 g body weight) and killed by decapitation. The brains were removed from adult mice and tissues containing the sub-ventricular zone (SVZ), were dissected out. Tissues derived from a single mouse were used to generate each culture. The dissected tissue was transferred to a phosphate buffer solution containing penicillin and streptomycin 100 U/mL each (Invitrogen, San Diego, CA, USA) and glucose (0.6%) at  $4^\circ \text{C}$  until the end of the dissection. We performed an enzymatic dissociation transferring the tissue to an Earl's balanced salt solution (EBSS) (Sigma-Aldrich, St. Louis, MO, USA) containing 1 mg/mL papain (27 U/mg; Worthington DBA, Lakewood, NJ, USA), 0.2 mg/mL cysteine (Sigma-Aldrich), and 0.2 mg/mL EDTA (Sigma-Aldrich), and incubated for 45 min at  $37^\circ \text{C}$  on a rocking platform. Tissues then were centrifuged at 123g and the supernatant was discarded. The pellet was re-suspended in 1 mL of EBSS and mechanically dissociated using an aerosol resistant tip (1000  $\mu\text{m}$  Gilson Pipette). Cells were re-suspended in 10 mL EBSS and centrifuged at 123g for 10 min. The supernatant was discarded and the pellet re-suspended in 200  $\mu\text{L}$  of EBSS. The pellet again was dissociated mechanically using an aerosol resistant tip (200  $\mu\text{m}$  Gilson Pipette). Cells were re-suspended in 10 mL of EBSS and centrifuged at 123g for 10 min. The supernatant was discarded and the pellet re-

suspended in 1 mL of chemically defined DMEM-F-12 containing FGF2 (human recombinant, 10 ng/mL; Peprotech, Rocky Hill, NJ, USA, or Upstate Biotechnology, Lake Placid, NY, USA) and EGF (human recombinant, 20 ng/mL; Peprotech). The cells were counted and plated at 3500 cells/cm<sup>2</sup>, the spheres formed after 5–7 d were harvested, collected by centrifugation (10 min at 123g), mechanically dissociated to a single-cell suspension, and re-plated in medium containing the appropriate GF(s) (19).

This procedure was repeated every 3–5 d (passage) *in vitro* for up to 12 months. The total number of viable cells was assessed at each passage by Trypan blue exclusion. Stem cells used in these experiments were between the fifth to the fifteenth passage in culture.

### Differentiation Assays

The differentiation of the NSCs was performed by plating the dissociated neurospheres in a medium containing 1% fetal calf serum and adhesion molecules (Matrigel), and without growth factors (EGF and bFGF), in a density of 40,000 cells/cm<sup>2</sup>. The differentiation was achieved at 7 d at  $37^\circ \text{C}$  5% CO<sub>2</sub> (19).

### Recovery of GFP-Positive Cells from Injured Transplanted Spinal Cord

NSCs were obtained from a transplanted spinal cord, taking the 8 mm region spanning the lesion site (for NSC preparation see the previous paragraph and 19). NSCs from the lesion (which contained endogenous spinal cord NSCs and transplanted GFP-NSCs) were cultured and passaged three times. In order to exclude the auto-fluorescence of the neurospheres, cells were dissociated and plated as single cells on Matrigel-coated glass coverslip. After 2 h, the cells were fixed and stained using an antibody against GFP. This experiment of recovery was repeated twice.

### Fibroblast Preparation and Culturing

Fibroblasts were prepared from skin and cultivated as described elsewhere

(21); briefly, the desired skin section was disinfected with 70% ethanol and the hair removed. The dermis was removed from the external part by scratching with a razor blade, and then washed with PBS.

The dermis was minced and incubated in PBS containing dispase 1.5 mg/mL (SIGMA T3417) and collagenase 0.15 mg/mL (SIGMA C9263) until the complete disintegration of the tissue.

The tissue then was centrifuged at 100g for 15 min at room temperature and resuspended in DMEM containing 10% FCS, and incubated at 37° C 5% CO<sub>2</sub> until the fibroblasts almost completely covered the surface of the flask.

### Preparation of Mouse Bone Marrow

The femurs and tibias were cut out and the muscles removed. The bones were placed into Hank's balanced salt solution (HBSS) to prevent their drying out. The ends of the bones were cut and the bone was flushed into a 50 mL conical tube with HBSS using a short (5/8") 25 G needle and a 10 mL syringe. The suspension was spun down at 400g for 5 min, resuspended in PBS, and washed again in PBS. The cells were counted in a hemocytometer and diluted to a concentration of 10,000 cells/μl for the transplantation.

### Chemically Killed GFP-NSCs

GFP-NSC neurospheres were dissociated and then spun down at 123g for 10 min. The supernatant was removed and 500 μl of 4% paraformaldehyde was added in the tube and the pellet was resuspended. The cells were kept for 10 min at room temperature, then added with 10 mL of PBS, spun down at 123g, and resuspended in PBS. This washing step was performed twice, and, finally, the cells were resuspended at 10,000 cells/μl in PBS.

### Spinal Cord Injury with the Infinite Horizon Device and the Treatment of the Mice

The traumatic SCI was performed using a commercially available Infinite

**Table 1.** Relative percentage of various markers indicative of differentiation of the NSCs *in vitro* and *in vivo* 3 wks and 3 months after transplantation, respectively.

Time	Nestin	βTub.III	SSEA-1	GFAP	Oligo	Neurofilaments
<i>In vitro</i> differentiation (1 wk)	3 ± 2 <sup>a</sup>	15 ± 2.1	0	75 ± 6	2 ± 1	14 ± 3
3 wks after administration i.v.	93 ± 2.0	94 ± 2.3	2 ± 1.1	3 ± 0.5	0	0
3 mo after administration i.v.	97 ± 1.7	94 ± 2.5	0	1 ± 0.7	0	2 ± 0.1

<sup>a</sup>The values indicate the mean ± SEM.

Horizon (IH) spinal cord injury device (17,22) at the T8 level. Surgery on the animals was performed as described elsewhere (17,18). A force of 50 Kdynes was applied for 1 s, then the animal muscles were sutured and clips were used to close the skin.

### Experimental Groups for Transplantation

We transplanted the animals by i.v. injection in the tail vein. The animals were divided into five groups: 1) Naïve mice not laminectomized and not lesioned; 2) Laminectomized mice; 3) Lesioned mice transplanted with phosphate buffer (PBS) by i.v.; 4) Lesioned mice transplanted with murine fibroblasts by i.v.; and 5) Lesioned mice transplanted with NSCs by i.v.

In another experiment, the animals were divided in three groups: 1) Lesioned mice transplanted with NSCs by i.v.; 2) Lesioned mice transplanted with NSCs by intraspinal injection; 3) Lesioned mice transplanted with PBS by intraspinal injection.

As additional controls, we used chemically killed GFP-NSCs and bone marrow by i.v.

### Cell Administration

NSCs, fibroblasts, or PBS were administered after spinal cord lesion. The first day, treatment was a slow i.v. injection of 50 μl in the tail vein performed within 30 min after injury, followed by a second injection 6 h later and a third injection 18 h after the lesion. Each cellular administration consisted of 330,000 cells resuspended in PBS for a total of 1,000,000 cells. The choice of a time limit of 18 h after SCI for administering NSCs is due to the optimal permeability of the blood

brain barrier at this time (23). In the case of intraspinal injection, we performed two injections of 50,000 cells (or the equivalent volume of PBS) rostrally and caudally at the site of the injury. Before the transplantation, the cultures were tested for proliferation and differentiation; only the cultures that showed a normal proliferation and differentiation capability (compared to a reference culture) were transplanted. The percentage of neurons, astrocytes, and oligodendrocytes in *in vitro* differentiation is reported in Table 1.

### Behavioral Testing

**Basso Mouse Scale.** All outcome measures were assessed in a blinded fashion. Neurological function was evaluated first 24 h after injury and then twice a week for the first 4 wks. The methods utilized are well known in the field of behavioral evaluation of recovery of function after SCI. Locomotor function and hind limb recovery after contusion were evaluated with the open field test according to the Basso mouse rating scale (24). We used nine animals per group in the behavioral experiments.

Allodynia-like responses in the unaffected forepaw were assessed by means of standard hotplate test and cold stimulation. For hotplate testing, mice were placed on hotplate and the latency to licking was measured. Non-responders were removed after 60 s. The response to cold was tested by the application of ethyl chloride spray to the palm surface. The response was rated 1 (no response), 2 (brief withdrawal with licking), and 3 (vocalization, withdrawal with licking, and aversion) (25). Animals, unless used for histology, survived for more than 5 months after the lesion.

## Histology and Immunocytochemistry

At the end of the experimental period, euthanasia of the animals and the preparation of the sections for histology and immunocytochemistry analysis was performed as described elsewhere (17,18). Briefly, primary antibodies for nestin (1:80),  $\beta$ -tubulin III (1:80), ED-1 (1:80), CGRP (1:500), GFP (1:1000) (Chemicon, Temecula, CA, USA), SSAE1 (stage specific embryonic antigen 1) (1:100; R&D Systems, Minneapolis, MN, USA), and GFAP (1:100; Sigma-Aldrich, St. Louis, MO, USA), were applied overnight at 4° C, and then incubated for 3 h at room temperature with fluorescein isothiocyanate or rodamine (conjugated rabbit anti-mouse antibody (1:70; Chemicon), or Alexa 488 and Alexa 546 (1:200) anti-mouse or anti-rabbit antibody (Invitrogen, Carlsbad, CA, USA). Alternatively, lesion cords were frozen quickly, stored at -80° C, and sectioned by means of a cryostat (Microm HM 500, Zeiss, Jena, Germany). Every twentieth section was stained with hematoxylin and eosin.

One cross-section containing the lesion epicenter and the total T8 segment cavitations were analyzed with computer-assisted image analysis. The percent cavitation was calculated as the area of cavitated tissue divided by the area of the total cross-section at the level of the injury (seven animals per experimental group were used for these experiments). Vibratome sections (40  $\mu$ m thick) also were collected on glass slides, alternatively, 10  $\mu$ m thick cryosections were collected on glass slides (see specific figures).

## Fluorescent Cells Counted in the Region of the Lesion

We considered GFP-positive cells that resulted intensely fluorescent. Briefly, in a fluorescence microscope, a cryosection obtained from the spinal cord of animals treated with fibroblast (not GFP-fluorescent) or PBS were used as reference (zero fluorescence), lowering the intensity of the emission of the lamp until they resulted negative, with this reference, we analyzed the GFP-NSC treated animals, noting all cells all that

resulted GFP-fluorescent with these parameters as positive.

The counting of the cells was performed assessing the GFP-positive cells in the transversal sections in a region of 4 mm centered in the lesion. The GFP-positive cells present in a group of three consecutive sections (10  $\mu$ m thick) were averaged, and we repeated this count (each 400  $\mu$ m). The total number was extrapolated by non-linear regression using Gaussian equation that showed a correlation greater than 0.90.

## Apoptosis

Apoptosis of oligodendrocytes within the *fasciculus cuneatus* was determined 14 d after SCI as described elsewhere (17,18). Eight animals per group were used in the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) experiments.

## Confocal Analysis

Images were taken using a Leica SP2 microscope with He/Kr and Ar lasers (Heidelberg, Germany).

## Tissue Isolation for RNA Preparation from Lesioned and Unlesioned Mice

Mice were anesthetized (17,18) and killed by decapitation. Laminectomy was performed at the T5–T12. The spinal cord region corresponding at the lesion site was removed (we took 4 mm of tissue rostral and 4 mm of tissue caudal to the lesion center). The tissue was put in 1 mL of TRIZOL Reagent (Invitrogen), shock frozen, and kept at -80° C until performing the RNA isolation. We used five animals per group for this analysis.

## RNA Isolation and DNase I Treatment cDNA Synthesis

These procedures were performed following the manufacturer's instructions. Total RNA was isolated using Trizol Reagent (Invitrogen) in accordance with the manufacturer's instructions. The genomic DNA was removed by DNase I treatments (2 U/ $\mu$ g of RNA) (Ambion, Austin, Texas, USA). The synthesis of single-strand cDNA was carried out on

1  $\mu$ g of RNA, using U M-MLV Reverse Transcriptase III (Invitrogen) following the manufacturer's instructions.

## SYBR Green Real-Time PCR

Real-time (RT)-PCR was performed in an MJ Opticon 2 using Brilliant SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA, USA) following the manufacturer's instructions. After a cycle of 95° C for 10 min, the reactions were cycled 40 times under the following parameters: 95° C for 30 s, then 56° C for 1 min, and 72° C for 1 min. A non-template control (NTC) was run in order to exclude the presence of genomic DNA, and all experiments were performed three times.

## PCR Data Analysis

We performed an analysis using the  $\Delta\Delta C_t$ , this procedure can be used since we have determined previously that the replication efficiencies (slopes of the calibration or standard curves) for the genes of interest and housekeeping gene are very close. The selection of the primers was performed using a DNASTAR Laser-gene program.

## Primers Used for Real-Time PCR

BDNF (product length: 158 bp); BDNF F 5'-cattactctctcatctgttg-3'; BDNF R 5'-cgtggagctttactctttcatgg-3'; CNTF (151 bp); CNTF F 5'-acatcagctctcccggtggcattag-3'; CNTF R 5'-tctcctgtggctttggggttc-3'; IL-6 (169 bp); IL-6 F 5'-gacaaccacggcctccctac-3'; IL-6 R 5'-cggtgtcacaatcagaattgcc-3'; LIF (101 bp); LIF F 5'-aacgtggaaaagctatgtgcg-3'; LIF R 5'-gcgaccatccgatacagctc-3'; NGF (165 bp); NGF F2 5'-tgggccaataaaggtttg cc-3'; NGF R2 5'-tgggcttcaggacagagtc tcc-3'; NT3 (109 bp); NT3 F 5'-acgtccctgg aatagtacacg-3'; NT3 R 5'-ttggatgcca cggagataagc-3'; TNF $\alpha$  (100 bp); TNF $\alpha$  F 5'-tctatggccagaccctcacac-3'; and TNF $\alpha$  R 5'-cagccactccagctgctcctc-3'.

## Statistical Analysis

Data are expressed as the mean (AV)  $\pm$  Standard Error Mean (SEM). We performed an analysis of variance (ANOVA) test followed by Bonferroni's post-test



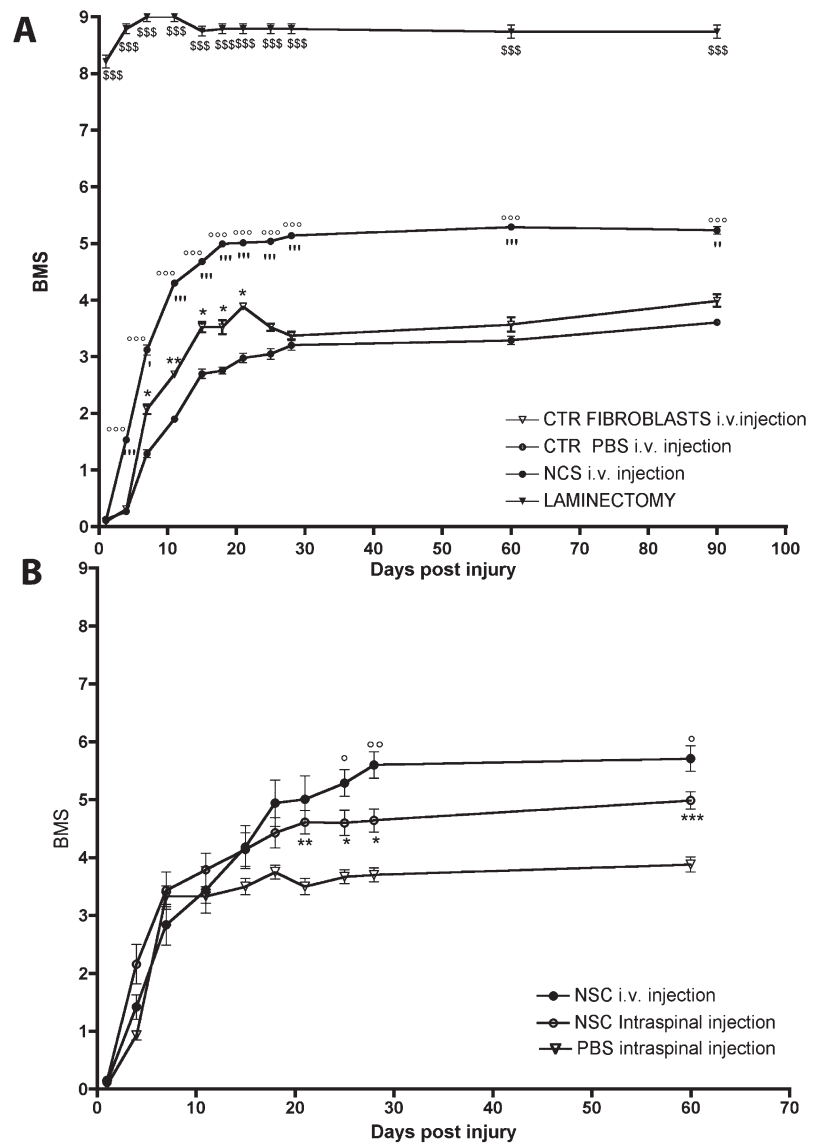
for the real-time PCR study and the behavioral analysis, and a *t* test for the quantification of the spare tissue at the lesion site. Statistical significance was accepted for *P* < 0.05.

**RESULTS**

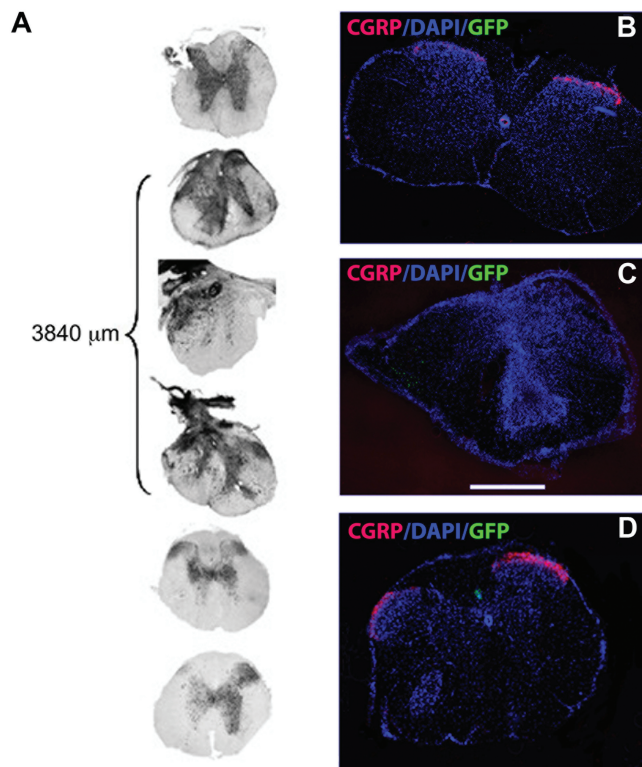
**NSCs Improve Recovery of Hind Limb Function**

Following the 50 Kdyne traumatic lesion to the cord, there is a total loss of ability in using hind limb as shown by the motor score (BMS scale) (Figure 1), this is followed by a gradual recovery that reaches its maximum extent (3.20 ± 0.08 [average ± SEM], *n* = 9 points of the BMS scale) in 4 wks (Figure 1A,1B) which corresponds to plantar placing of the paw with or without weight support or occasional, frequent, or consistent dorsal stepping, but not plantar stepping. When lesioned mice were treated with adult NSCs administered by i.v. (see Materials and Methods), the recovery was much faster and higher (up to 5.14 ± 0.06 at d 28, *n* = 9), corresponding to frequent or consistent plantar stepping without coordination, or frequent or consistent plantar stepping with some coordination, but with the paw rotated at the initial contact and lift off. This was particularly evident between d 4 and 15 after SCI, and was maintained throughout the experimental period (up to 90 d, *n* = 9). When, in a different experiment, NSCs were applied by intraspinal injection, the extent of recovery was smaller compared to the i.v. application, although the outcome was significantly better than saline throughout the experimental period (see Figure 1B). The groups were randomized, the analysis was performed in double blind fashion, and each group was composed by nine animals. No differences with the control (PBS, i.v.) group were found in the animals transplanted with killed NSCs or bone marrow cells.

No signs of allodynia-like forelimb hypersensitivity were recorded (25) in our lesioned mice at any time throughout the observational period of 90 d after SCI with NSCs applied via i.v. or intraspinal injections. These behavioral results corre-



**Figure 1.** (A) Effect of PBS, fibroblasts, and NSC i.v. treatments on locomotor activity after SCI in open field. Locomotor recovery was evaluated over a 3-month period, using the nine-point BMS scale. Values represent mean ± SEM. We analyzed the statistical differences with the ANOVA test followed by Bonferroni's post-test method. ▼ Laminectomized mice; ● NSC-treated animals; ▽ Fibroblast-treated mice; ○ PBS-treated mice. Significance symbols: \$, Laminectomized mice versus all other groups; ', NSC-treated mice versus fibroblast-treated mice; °, NSC-treated mice versus PBS-treated mice; \*, Fibroblast-treated mice versus PBS-treated mice. \$\$\$,°°°,''',\*\*\**P* < 0.001; \$\$,°,''',\*\**P* < 0.01; \$,°,',\**P* < 0.05. BMS, Basso Mouse Scale (nine animals per group were used in this analysis). (B) Effect of NSC administration via i.v. route or intraspinal injection on open field locomotor activity after SCI. Hind limb recovery of function was evaluated over a 2-month period using the nine-point BMS scale. Values represent average ± SEM. We determined the statistical differences by means of an ANOVA test followed by Bonferroni's post-test. For this experiment the number of animals was six per group. ● Animals treated with NSCs by i.v. injection; ○ Animals treated with NSCs by intraspinal injection; ▽ Animals treated with PBS by intraspinal injection. °, NSC i.v.-treated mice versus NSC intraspinal-transplanted mice; \*, NSC intraspinal-transplanted mice versus PBS intraspinal-treated mice; °°°,\*\*\**P* < 0.001; °°,\*\**P* < 0.01; °, \**P* < 0.05.



**Figure 2.** CGRP immunolocalization at various distances from the lesion 3 months after the transplantation, indicating the lack of changes in the classical distribution of CGRP-positive immunolabeling of the dorsal horn at the margins of the lesion site in the cord of NSC-treated mice compared with more rostral or caudal sections outside the injury. (A) Three sections within 4 mm of the spinal cord region encompassing the lesion are shown; (B) rostral to the lesion; (C) at the site of the lesion; (D) caudal of the lesion. CGRP-positive cells are shown in red; the transplanted GFP cells are shown in green; DAPI staining of the nuclei is indicated in blue. Comparing the rostral (B) and caudal (D) section to the section corresponding to the lesion (C), we can see a strong reduction of CGRP staining. Scale bar 10  $\mu\text{m}$ .

late with the lack of changes in the classical distribution of CGRP-positive immunolabeling of the dorsal horn at the margins of lesion site in the cord of NSC-treated mice (Figure 2) compared to more rostral or caudal sections outside the injury.

Fibroblast administration with the same procedure utilized for NSCs induced, between 7 and 21 d, a transitory recovery of hind limb function significantly better than the saline, but was not maintained at later stages (see Figure 1A).

In a parallel experiment, we compared the motor recovery between lesioned mice transplanted with NSCs by i.v. and intraspinal injection. We noticed that

there was a significant difference between the i.v. and intraspinal injection from 25 d post-transplantation up to 2 months post-transplantation, and there was a significant difference between the mice transplanted with NSC or PBS by intraspinal injection from d 21 up to 2 months. (see Figure 1B).

#### NSC-Mediated Reduction of Post-Traumatic Cavity and Oligodendrocyte Apoptosis

The post-traumatic scar is evident in saline-treated lesioned mice (Figure 3), and its size is markedly reduced by NSC application, which reduces the CNS-spared tissue outside the scar (Table 2).

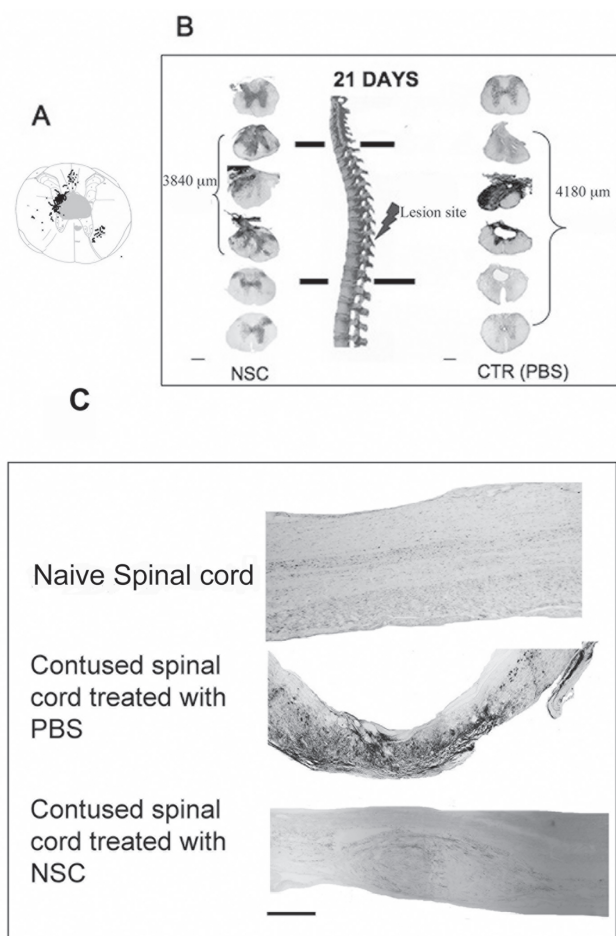
Serial sectioning was performed throughout the site of injury of seven spinal cords per experimental group.

TUNEL labeling, 2 wks after injury in the *fasciculus cuneatus* in 10  $\mu\text{M}$ -thick sections 2.5 mm rostral to the lesion epicenter, revealed a mean number of TUNEL-positive cells of  $22.9 \pm 1.2$  for saline-treated animals, whereas, for animals treated with NSCs, the number of TUNEL-positive cells was reduced markedly to  $6.9 \pm 0.3^{***}$  ( $n = 8$  each group;  $^{***}P < 0.001$ ).

#### NSC Homing to Site of Injury, Survival, and Antigen Expression

GFP-labeled NSCs (Figure 4A) are accumulated mainly in the gray matter at the lesion site with a small presence in the white matter. We serially sectioned seven cords, and the average number of GFP-positive cells was 20,000 per NSC-transplanted spinal cord, representing about 2% of injected cells. At 3 wks after cell administration, most engrafted NSCs were positive for nestin and  $\beta$ -tubulin III, and only a very small number expressed the stem cell marker SSEA1 (Figure 4B, 4C, 4D, Table 1). In addition, very few ED1-positive cells (macrophages) were present at the site of the lesion and we did not detect any phagocytosis of NSCs at this time (representative sections are shown in Figure 5A, 5B, 5C). On the contrary, macrophages were abundant at the site of lesion 5 months after the transplantation, and had phagocytated most of GFP-positive cells (representative sections are shown in Figure 5D, 5E, 5F). At 5 months after the transplantation, it appears that there are more GFP-positive cells in respect to the GFP-positive cells found at 1 month (Figure 5). An explanation of this evidence is that macrophages that have phagocytated GFP cells can accumulate in some region of the lesion, whereas the live GFP-NSC are more distributed across the lesion. Very few cells were co-labeled for GFP-protein and GFAP, and were located close to the CNS glial scar (see Table 1).

One week after transplantation, GFP-NSCs (i.e.) still maintained the capacity



**Figure 3.** Histological analysis of the spinal cord 21 d after lesion by tionine staining. (A) Schematic drawing of the transverse spinal cord at the level of the lesion in NSC-transplanted cells animals. The green fluorescent cells transplanted by endovenous injection, and which reached the site of the lesion, are indicated in black. The lesion is represented by the central gray region. (B) Histological comparison between NSC-treated mice (left) and PBS-treated mice (right) 21 d after T8 contusion. Serial transverse cryostat sections (10 μm) were performed within a 4 mm region encompassing the site of the lesion and stained with tionine. Scale bar 500 μm. (C) Longitudinal cryostat section (10 μm) of a naive (top), contused PBS-treated (middle), and contused NSC-treated spinal cord stained with tionine. The lesioned cord from PBS-treated mice is bent because of the lack of tissue at site of injury and, once placed in paraffin, it cannot maintain a straight alignment. This does not happen with lesioned cords of NSC-treated mice and is a further macroscopic evidence of the effect of the treatment. Scale bar 500 μm.

to produce neurospheres when recovered from the lesion site and grown *in vitro*. We applied to the spinal cord lesion site the same procedures used to obtain the cells from CNS sub-ventricular zone; GFP-NSCs were isolated 1 wk after their transplantation and re-grown *in vitro* to re-form neurospheres (Figure 6). The per-

centage of GFP-NSCs recovered in this experiment is  $36 \pm 2\%$ . This result may not represent the true fraction of GFP-NSCs present in the lesion, since it is the ratio between the total number of recovered spinal cord stem cells (thus comprising also the endogenous ones) and the GFP-NSCs after three passages in

**Table 2.** Quantification of the Spared Tissue 3 months after Lesion<sup>a</sup>

Treatment	Lesion Epicenter	T9
Vehicle	$60.0 \pm 5.1^b$	$71.0 \pm 2.7^b$
NSCs I.V.	$88.6 \pm 3.4^{b,c}$	$91.1 \pm 2.4^{b,d}$

<sup>a</sup>Serial sectioning was performed throughout the site of injury of seven spinal cords per experimental group. The table indicates the percentage of CNS spare tissue outside the scarring tissue at the lesion site (T8) in the control (PBS) or in the NSC-treated animals (column 2) or at the level of T9 (column 3).

<sup>b</sup>The values indicate the mean  $\pm$  SEM.

<sup>c</sup> $P < 0.01$  vehicle versus NSCs.

<sup>d</sup> $P < 0.001$  vehicle versus NSCs.

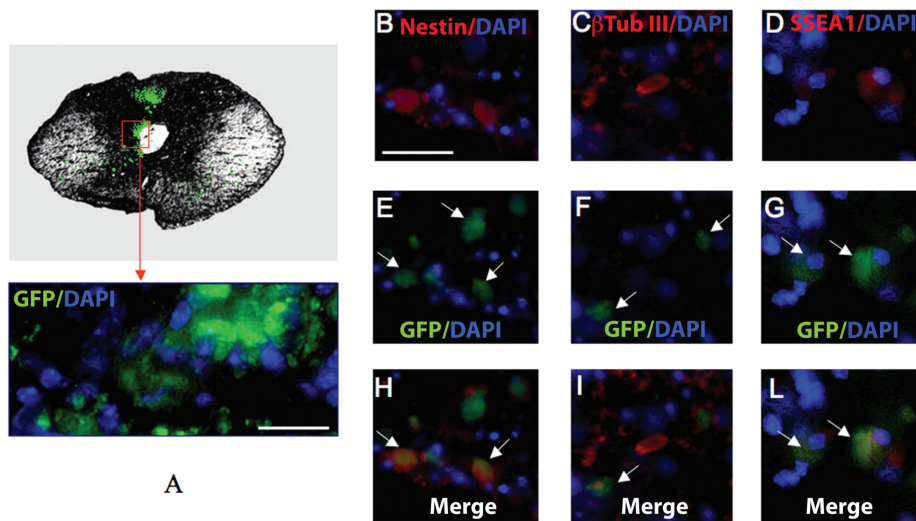
culture (2 wks in culture). This difference also may be the result of diverse proliferation rates between the two populations of stem cells.

### NSC-Mediated Chemokine and Growth Factor Expression in the Injured Cord

The prompt action of NSCs in promoting the recovery of function was suggestive of some additional effects by these cells beyond their cellular presence in the injured cord. Accordingly, we have assayed the production of neurotrophic factors and inflammatory cytokines that represent the major targets of pharmacological agents aimed at repair in neurodegenerative disorders (17,18,26). The evaluation was performed at the site of injury (T8 segment) 1, 2, and 7 d after NSC administration.

We studied the mRNA levels of seven different factors (Figure 7), namely brain-derived neurotrophic factor (BDNF); ciliary neurotrophic factor (CNTF); neurotrophin 3 (NT3); nerve growth factor (NGF); tumor necrosis factor  $\alpha$  (TNF $\alpha$ ); interleukin-6 (IL-6); and leukemia inhibitory factor (LIF). The comparison was made between the animals treated with PBS, fibroblasts, or NSCs. As an additional control, we used the T8 region of the spinal cord of naive unlesioned animals. Each group was composed of five mice, and we performed three quantitative (Q)-RT-PCRs per each

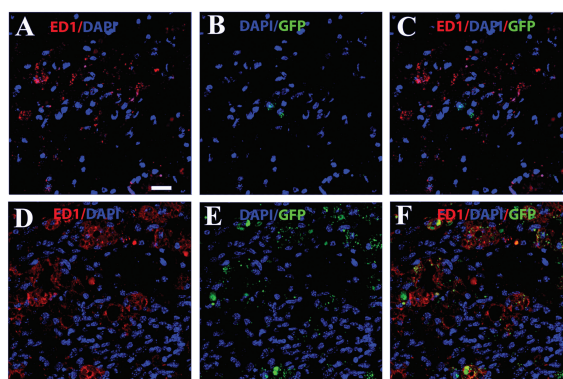




**Figure 4.** Immunolocalization of the Nestin,  $\beta$ -Tubulin III, and SSEA1-positive cells at the site of the lesion 3 wks after the contusion. Serial cryostat transversal section 10  $\mu$ m thick. (A) GFP-positive cell (green) localization 3–4 wks after i.v. transplantation in a transversal section of a spinal cord; (B) Nestin staining (red); (C)  $\beta$ -Tubulin III staining (red); (D) SSEA1 staining (red); (E–G) Green fluorescence (in green) (indicated by the arrows) of the section corresponding respectively to B, C, and D; (H–L) Merge of the section B–E, C–F, and D–G (the arrows indicated the double positive cells). Scale bars 10  $\mu$ m are shown only in A and B. The nuclei (4,6-diamidino-2-phenylindole dihydrochloride (DAPI)-positive) are indicated in blue; many GFP-positive cells have the nuclei labeled with DAPI.

time point (in each experiment, we run a duplicate per each sample and a parallel PCR for the gene of interest and the reference gene Glyceraldehyde-3-Phosphate Dehydrogenase).

CNTF was unmodified by either lesioning or NSCs, while BDNF and NT3 were practically unchanged by the lesion and very significantly increased by NSCs application. NGF and LIF were increased



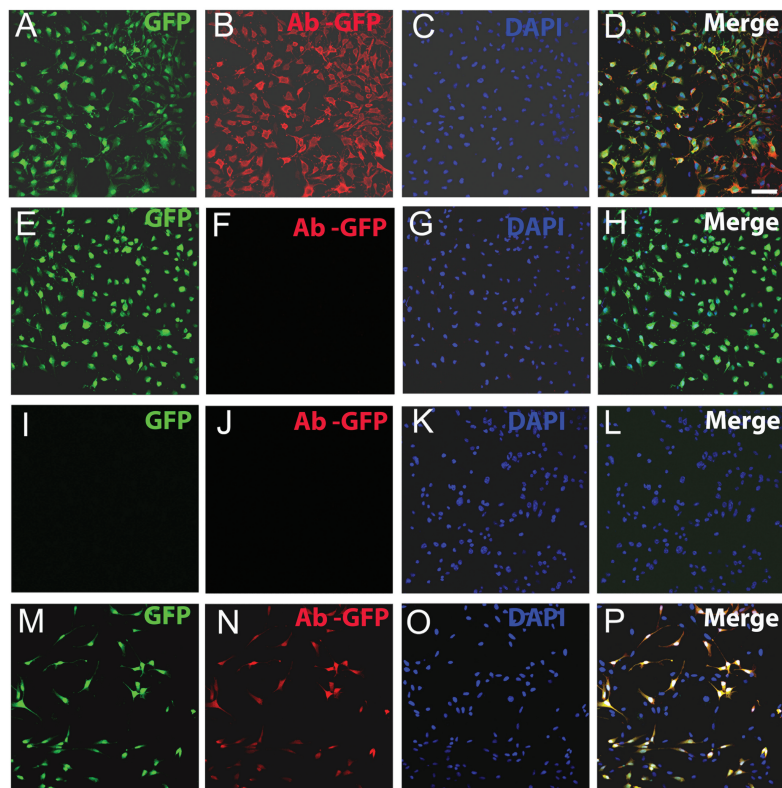
**Figure 5.** Confocal analysis of the transverse section at the site of the lesion. Staining for ED1 (macrophage staining) (in red); GFP-fluorescent cells (in green); DAPI nuclei staining (in blue). (A–C) 3 wks after the transplantation. (D–F) 5 months after the transplantation. Scale bar 20  $\mu$ m in A is representative for all the pictures.

by the lesion and far more so by NSCs treatments. The inflammatory cytokines IL-6 and TNF $\alpha$  were increased by the lesion, but the latter was increased markedly by NSC treatments. All the above effects were restricted to 48 h after NSC administration; no changes from control values were observed at 24 h and 7 d (see Figure 7) in the NSC-treated mice.

## DISCUSSION

In this study, it is shown that treatment of spinal cord injury with adult brain-derived NSCs attenuates secondary degeneration and improves recovery of motor function with no allodynia in unaffected forepaws. About 2% of the 1,000,000 i.v.-administered NSCs homes for the site of injury, where they are located mainly in the gray matter and remain morphologically undifferentiated. Almost all NSCs are positive for nestin and  $\beta$ -tubulin III, and very few for GFAP. These results corroborate previous observations reporting that nestin is expressed by immature neurons in culture (those, for instance, that are  $\beta$ -tubulin positive [27] and adult human brain cortical neurons [Gorio *et al.*, unpublished laboratory notes]). Live GFP-labeled NSC were recovered from the lesion cord at 7 d after administration and regrown as neurospheres *in vitro* (see Figure 6). Several GFP-positive NSCs were surrounded by ED1-positive cells, and, later, all NSCs were phagocytated (see Figure 5). The number of NSCs in the lesioned area is very small compared to the amount of tissue and the number of cells involved in the injury process, thus, it is very unlikely that the rather quick recovery of functions (maximum extent of permanent recovery reached within 3–4 wks) may be ascribed to a cellular substitution of the lost tissue. This also is supported by the fact that an average of only 20,000 i.v.-administered NSCs reached the injured region of the cord and promoted a greater recovery than 100,000 cells applied intraspinaly at the lesion site. A possible role in such a recovery may be ascribed to the prompt marked increase in neurotrophic factor expression trig-





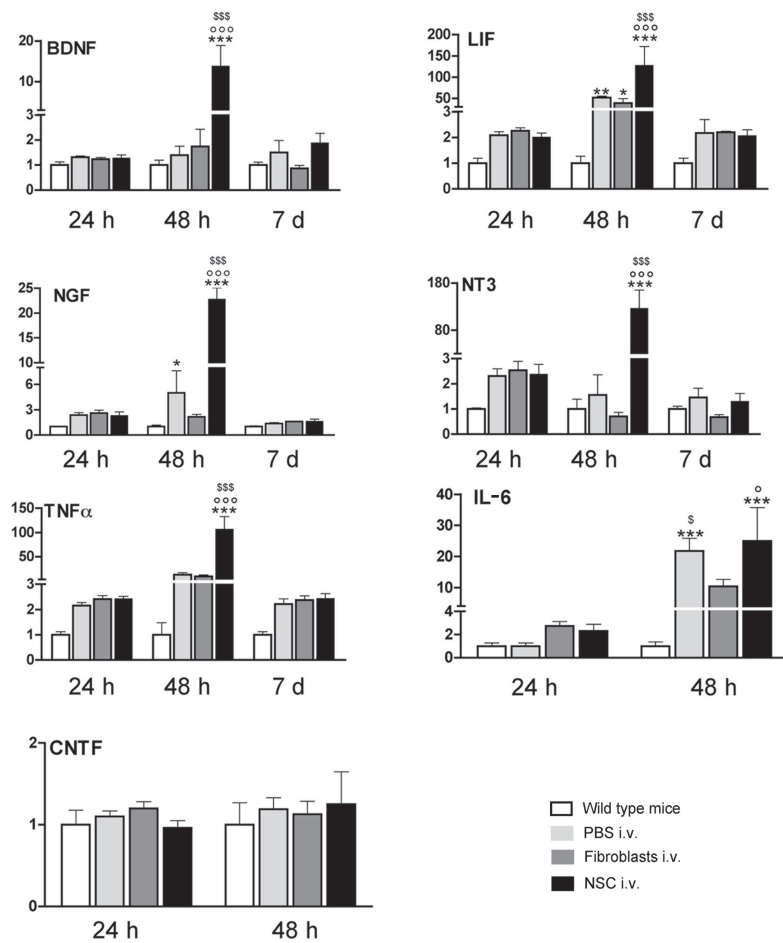
**Figure 6.** Confocal analysis of the GFP-NSCs recovered 1 wk after transplantation. A, B, C, D show GFP-NSCs grown in culture as positive control (A) and the antibody staining to GFP (using Alexa 546 labeled secondary antibody) labels all the cells (B). Merge in D, all cells are positive. E, F, G, H show GFP-NSCs grown in culture as positive control (H) and no antibody to GFP labels was added (F) (negative control for the antibody staining). Merge in H all cells are, however, green positive, but not stained for the antibody. I, J, K, L show wild-type NSC obtained from a non-GFP-wild type mouse presented as negative control. Negative in fluorescence for GFP and no labeling with antibody to GFP (I and J). The nuclei show the presence of stem cells (K and L), but labeling is lacking. M, N, O, P show GFP-NSCs recovered from lesion site 1 wk after i.v. administration (M) and positive to anti-GFP-antibody (N); the nuclei are more numerous (O), indicating presence of non-GFP-NSCs from the cord. Merge (P) indicates that 36% of these stem cells are transplanted cells. C, G, K, and O show DAPI staining of the nuclei. Scale bar 50  $\mu$ m in D is representative for all the pictures.

gered by NSCs (see Figure 7); this also may have contributed to the attenuation of secondary degeneration. It is known that local BDNF administration may reverse SCI-induced neuronal cell body atrophy in the red nucleus (28), and, similarly, NT-3 is capable of counteracting axotomy-induced death of corticospinal neurons (29). In addition, NT-3 and BDNF effectively prevent the degeneration of ascending sensory fibers (30). In agreement with our results, the favorable

neurotrophic effect of neurotrophins was not accompanied by axonal regeneration or sprouting (31). The enhanced expression of trophic factors at the site of injury may not derive directly from NSCs, since assays on cultured NSCs revealed only a very low production (32). It is, however, possible that the interaction with the lesion environment may have enhanced their ability to produce trophic factors (33). NSCs mediated, also, a hundred-fold increase of TNF $\alpha$  with no effects on

lesion-induced increase of IL-6. NSCs in culture do not produce TNF $\alpha$  (34). Thus, the posttraumatic neuroinflammatory reactions are affected by NSCs in a rather complex manner. TNF $\alpha$  is known to be involved in CNS myelination and demyelination and one of the reported side effects of the therapies with anti-TNF $\alpha$  agents is the onset of the typical symptoms of demyelinating diseases such as multiple sclerosis. Thus, the improved recovery also may be secondary to a TNF $\alpha$ -induced effect upon remyelination of spared axons at the site of injury (35–37). This is supported by the reduced rate of recovery of function after SCI observed in mice carrying a gene knockout that blocks either TNF $\alpha$  production or TNF $\alpha$  receptor expression (37,38). Surprisingly, the transplantation of the injured mice with the fibroblasts induced a significant transitory recovery of the motor function compared to the PBS-treated mice. This effect was limited for the first 3 wks and was lower than the effect induced by GFP-NSC, perhaps because they died within three weeks after transplantation. In addition, the fibroblast treatment did not induce any change in cytokine expression. Exogenous GFP-NSCs still could be recovered live from the injured spinal cord the week after transplantation, and, in this early period, most of the recovery of function and the enhancement of chemokine and tropic factor expression was observed. In a control series of experiments, we used chemically killed NSCs, and no effect on recovery function was observed (data not shown). This suggests that only live NSCs might be able to interact positively with the lesion environment of the lesion cord and to promote recovery.

Hofstetter and collaborators (25) observed sprouting of CGRP-immunoreactive fibers and corticospinal tract axons after transplantation of naïve spinal cord-derived adult NSCs into the lesioned spinal cord. This was correlated with development of allodynia-like hypersensitivity in the forepaw. The excessive sprouting and allodynia was pro-



**Figure 7.** Real-time PCR of the expression of different cytokines. Five mice per group were used for this analysis. Bars indicate the fold difference of RNA levels in treated animals compared with control untreated naïve animals  $\pm$  SEM. 24 h, 48 h, and 7 d indicates time after transplantation that the analysis was performed. White bars: wild type animals; light gray bars: PBS-treated animals; dark gray bars: fibroblast-treated animals; and black bars: NSC-treated animals. Significance symbols: \$, versus fibroblast-treated mice; °, versus PBS-treated mice; \*, versus naïve mice. The symbols of significance relative to one treatment versus another were put on top of the bar corresponding to the sample for which the comparison was made. \$\$\$, °°°, \*\*\* $P$  < 0.001; \$\$, °°, \*\* $P$  < 0.01; \$, °, \* $P$  < 0.05.

portional to the number of grafted NSCs that differentiated into astrocytes. Most studies applying neurosphere-derived neuronal precursor cells to *in vivo* experimental conditions reported their differentiation in GFAP-positive cells as it occurs in *in vitro* differentiation experiments (15,17,25,39). It is possible that, in our study, the reduced number of graft-derived GFAP-positive astrocytes (see Table 1) may have hampered sprouting and the development of allodynia hyper-

sensitivity of unaffected forepaws. Whether this resilience to *in vivo* differentiation is the result of our preparation procedures or whether it is due to the posttraumatic inflammatory-hypoxic environment or the combination of the two, remains to be established. This latter hypothesis is supported by the observations of Romanko *et al.* (40), where it is shown that NSCs of the subventricular zone are resilient to hypoxic/ischemic insults, while progenitors die. Thus, it is

possible that the posttraumatic environment may have caused the selection of an NSC subpopulation that survived at the unfavorable site of injury and lacked the ability to differentiate. Our results obtained with adult NSC differ also from those obtained with fetal cells by Lu *et al.* (41). They described an increase where the enforced expression of neurotrophic factors was accompanied by extensive axonal sprouting and growth in the injured cord.

While chemically killed and whole bone marrow cells did not affect the rate of recovery after SCI (data not shown), fibroblasts showed an early fluctuating positive effect with respect to the PBS-treated mice that diminished (see Figure 1) within the first 3 wks. Their effect was much lower compared to the outcome induced by GFP-NSC, perhaps because the fibroblast survived in the hostile environment of the lesion for a lesser time than GFP-NSC. In addition, the fibroblast treatment did not induce any significant change in cytokine expression that would justify this transient effect (see Figure 7).

In conclusion, our study shows that cellular therapy with adult NSCs via an *i.v.* may represent a useful treatment for spinal cord injury; the early attenuation of secondary degeneration is followed by an enhanced recovery of function that is maintained up to 5 months after NSC administration, although, at this stage, most NSCs are phagocytated by ED1-positive cells (see Figure 5). At 5 months after the transplantation, it looks appears there are more GFP-positive cells (see Figure 5). This can be due to the accumulation of the macrophages that have phagocytated GFP cells in some region of the lesion, whereas the live GFP-NSC are more distributed across the lesion. NSCs home for the site of injury do not differentiate, and can be recovered from the injured cord and regrown *in vitro* within 1 wk after SCI. These cells are able to modify the environment of the injury site by increasing the expression of trophic factors and TNF $\alpha$  greatly.

**ACKNOWLEDGMENTS**

We acknowledge the continuous support by Drs Guglielmo Brayda, Fabio Colbucci, Mauro Menarini, Roberto Piperno, Maria Taricco, Giulio Cicognani, of the Scientific Advisory Board of the Fondazione Montecatone. We would like to thank Raffaella Adami for the confocal images.

The project was supported by a grant from The Italian Ministry of Education in 2003 and in 2005, and by a generous donation from the Montecatone Foundation, Imola (It) to Alfredo Gorio.

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