

NEUROSYSTEMS

Embryonic and adult stem cells promote raphespinal axon outgrowth and improve functional outcome following spinal hemisection in mice

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Abstract

Spinal cord injury (SCI) often results in permanent neurological deficits below the injury site. Serotonergic raphespinal projections promote functional recovery after SCI, but spontaneous regeneration of most severed axons is limited by the glial cyst and scar that form at the lesion site. Stem cell (SC) transplantation offers a promising approach for inducing regeneration through the damaged area. Here we compare the effects of transplantation of embryonic neural precursors (NPs) or adult mesenchymal SCs, both of which are potential candidates for SC therapy. The spinal cord was hemisectioned at the L2 neuromer in adult mice. Two weeks post-injury, we transplanted neural precursors or mesenchymal SCs into the cord, caudal to the hemisection. Injured mice without a graft served as controls. Mice were tested for functional recovery on a battery of motor tasks, then killed and analysed for survival of grafted cells, for effects of engraftment on the local cellular environment and for the sprouting of serotonergic axons. Both types of SCs survived and were integrated into the host tissue, but only the NPs expressed neuronal markers. All transplanted animals displayed an increased number of serotonin-positive fibres caudal to the hemisection, compared with untreated mice. And both cell types led to improved motor performance. These results point to a therapeutic potential for such cell grafting.

Introduction

Traumatic spinal cord injury (SCI) results in loss of sensory and motor function caudal to the injury; currently, no effective treatment exists for mitigating the effects of such damage. Complete anatomical transection is rare and tissue bridges often persist across the lesion (Kakulas, 1999). The initial mechanical trauma, due to compression, distraction or laceration/transection, damages blood vessels and disrupts axon fibres and local neurons; it is followed by swelling, oedema, glial activation, oxidative stress, inflammation and cell death (Dumont *et al.*, 2001; McDonald & Sadowsky, 2002). Axon regeneration after SCI is limited: progressive tissue cavitation or glial cyst formation, associated with macrophage and microglial infiltration (Fawcett & Asher, 1999), and with the presence of cells expressing inhibitory molecules such as Nogo (Schwab, 2004) and presence of a glial scar due to reactive astrogliosis (McGraw *et al.*, 2001; Silver & Miller, 2004), contribute to this failure. Cellular, molecular and intense rehabilitative training therapies following SCI (Sadowsky *et al.*, 2002; Schwab, 2002; Garbossa *et al.*, 2006; Thuret *et al.*, 2006) may lead to mild repair and reorganization of intraspinal circuits (Fawcett & Asher, 1999; Bareyre *et al.*, 2004) and some growth of serotonergic fibres (Hashimoto & Fukuda, 1991).

Stem cell (SC) transplantation has the potential for promoting tissue repair and holds promise as a potent treatment for SCI (Barami & Diaz, 2000; Schwab, 2002; Fernandez *et al.*, 2006). Schwann cells, mesenchymal stem cells (MSCs), olfactory ensheathing cells, neural SCs, embryonic or adult stem/progenitor cells, genetically engineered SCs and activated macrophages have all been used in this regard (Fernandez *et al.*, 2006; Thuret *et al.*, 2006; Coutts & Keirstead, 2008): results from these studies are encouraging with regard to axonal regrowth and functional improvement (Schmidt & Jordan, 2000). Both embryonic and adult SCs have the capacity to integrate with host tissue, differentiate and provide neurotrophic support (Rabchevsky & Smith, 2001; Thuret *et al.*, 2006; Coutts & Keirstead, 2008).

Two types of SCs were utilized. (i) Neural precursors (NPs), including neuronal- and glial-restricted precursors, are harvested from embryonic neural tube (Mujtaba *et al.*, 1999); they have been shown to integrate into host tissue and promote regeneration and repair (Han *et al.*, 2004; Lepore & Fischer, 2005). In particular, they enhance the sparing and/or sprouting of serotonergic and noradrenergic fibres, and of axons positive for corticotrophin-releasing factor (Mitsui *et al.*, 2005). (ii) MSCs offer a growth-permissive substrate and may provide longitudinal directional guidance for host axons that are sprouting or regenerating (Hofstetter *et al.*, 2002; Ankeny *et al.*, 2004); MSCs can be collected from adult bone marrow, stored and expanded prior to transplantation; they have an immunomodulatory potential, thus reducing the need for immune suppression (Le Blanc, 2003; Spitkovsky & Hescheler, 2008).

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Here, we compare the effects of transplanting NPs or MSCs on axonal regrowth in a mouse model of SCI. We quantify the density of post-injury serotonin (5-HT)-positive fibres, and also evaluate functional outcome on a number of motor tasks. The results point to a significant potential for both types of SCs in enhancing spinal cord regeneration and in facilitating functional recovery. To our knowledge, this is the first report to compare the effects of transplantation of NPs and MSCs in the same experimental model and to show sprouting of raphe spinal axons following hemisection.

Materials and methods

Experimental animals

Two-month-old C57BL/6J male mice (Harlan-Italy, San Pietro al Natisone, Italy) were used for producing the SCI model. Animals had free access to food and water. All experimental procedures on live animals were performed according to the European Communities' Council Directive of 24 November 1986 (86/609/EEC) and University of Torino's institutional guidelines on animal welfare (DL 116/92) and were approved by the University of Torino's ethical committee; efforts were made to minimize the number of animals used.

BCF1 mice, which express enhanced green fluorescent protein (EGFP) under the beta-actin promoter, were kindly provided by Dr M. Okabe (Osaka University, Suita, Japan; Okabe *et al.*, 1997) and bred in our animal facility. Mice were killed by cervical dislocation and MSCs were harvested from bone marrow (see below). Pregnant mice at 12 days of gestation (E12) were killed by cervical dislocation and NPs for transplantation were obtained from the neural tube.

Isolation of NPs

EGFP-positive E12 embryos were removed and placed in chilled saline. Caudal neural tubes, corresponding to the ten lower somites, were mechanically dissected from the surrounding connective tissue and gently triturated with a Pasteur pipette; dissociated cells were collected by centrifugation, and observed under the microscope (Leitz Diavert, Wetzlar, Germany). Counts were made on a Burkert chamber, after incubation in 0.4% Trypan blue in PBS (phosphate-buffered saline); cells were re-suspended in saline at a final concentration of 50×10^3 cells/ μ L.

Immunofluorescence of cell cultures

Some cells were plated in 19.5-cm² polystyrene dishes (BD Biosciences, San Jose, CA, USA), pre-treated with a coating of poly-D-lysine hydrobromide (Sigma, St. Louis, MO, USA), at a concentration of 5000 cells/cm². Cells were cultured in complete medium (Mitsui *et al.*, 2005) comprising Dulbecco's modified Eagle medium F12, supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 μ g/mL streptomycin, 1 mg/mL bovine serum albumin (BSA), 20 ng/mL basic fibroblast growth factor, 10 μ L/mL N2 supplement, 20 ng/mL neurotrophin-3 (NT-3) and 20 ng/mL B27 supplement (all purchased from Invitrogen-Gibco, Carlsbad, CA, USA), in a humidified atmosphere of 95% air with 5% CO₂ at 37°C.

Cultured neural precursors were fixed with 4% buffered paraformaldehyde (PFA) supplemented with 4% sucrose for 30 min at 37°C. Samples were washed three times with PBS. In order to permeabilize cells, samples were eventually incubated for 10 min with PBS containing 0.25% Triton X-100 and then washed with PBS. After blocking unspecific binding sites with 1% BSA in PBS, cells were

incubated in the following diluted primary antibody in the same solution at 4°C overnight:

1. Polyclonal anti-gial fibrillary acidic protein (GFAP) (made in rabbit; 1 : 500; DakoCytomation, Denmark: catalogue no. Z 0334, raised against purified protein from isolated cow spinal cord): GFAP is the main intermediate filament specific for mature astrocytes in the central nervous system. On conventional Western blot analysis of adult mice brains, this antibody visualizes a single band at ~50 kDa, characteristic of GFAP (Garcia-Sevilla *et al.*, 2004).
2. Monoclonal anti-nestin (made in mouse; 1 : 20; Chemicon International Inc, Temecula, CA, USA: catalogue no. MAB353, clone name Rat-401, raised against nestin purified from embryonic rat spinal cord): according the manufacturer's technical information, the antibody reacts with rodent nestin protein (mouse and rat) but does not react with human protein; moreover, the rodent protein is recognized as a 200–220-kDa band in reducing gels of newborn rat or mouse cell extracts. This antiserum stains neuroepithelial cells including neural progenitors (Hockfield & McKay, 1985).
3. Monoclonal anti-microtubule-associated protein-2 (MAP-2) (made in mouse; 1 : 200; Chemicon: catalogue no. MAB3418, clone name AP20, raised against MAP-2 purified from bovine brain microtubule protein): the antiserum binds specifically MAP-2A and 2B, recognizing a double band of ~300 kDa, corresponding to microtubule-associated protein-2 (A and B) (Blanchart *et al.*, 2006).
4. Polyclonal anti-serotonin (5-HT) (made in rabbit; 1 : 1000; ImmunoStar, Hudson, WI, USA; catalogue no. 20080, raised against serotonin coupled to BSA with paraformaldehyde): this serotonin antiserum stains murine hypothalamus, raphe nuclei and spinal cord (Mitsui *et al.*, 2005).
5. Monoclonal anti-synaptophysin (made in mouse; 1 : 150; Immunological Sciences, Rome, Italy; catalogue no. MAB-10321, clone name SY 38, raised against synaptophysin prepared from presynaptic vesicles of bovine brain): synaptophysin is a transmembrane glycoprotein of presynaptic vesicles of brain, spinal cord, retina and neuromuscular junctions. It reacts specifically on Western blots with the 38-kDa synaptophysin band of rat and bovine brain and labels presynaptic terminals in sections prepared from a variety of mammalian tissues (Wiedenmann & Franke, 1985).
6. Polyclonal anti-myelin basic protein (MBP) (made in rabbit; 1 : 200; Immunological Sciences; catalogue no. AB-10025; raised against human MBP from brain); this antiserum labelled the major myelinated pathways of the brain, such as the corpus callosum and the internal capsule, and the white matter of the spinal cord (Forni *et al.*, 2006).
7. Mouse monoclonal anti-vimentin (1 : 200; DakoCytomation: catalogue no. M 7020, clone name Vim 3B4, raised against vimentin purified from bovine eye lens): this antibody reacts with vimentin, the 57-kDa intermediate filament protein present in cells of mesenchymal origin, as shown by the Western blot analysis performed by Wyatt *et al.*, 1991.
8. Monoclonal anti-neuronal nuclei (NeuN) (made in mouse; 1 : 10; Chemicon: catalogue no. MAB377; clone name A60, raised against purified cell nuclei from mouse brain): MAB377 specifically recognizes several bands corresponding to NeuN (46–48 kDa and possibly 66 kDa) in immunoblotting analysis of mice brain (Mullen *et al.*, 1992; Collombet *et al.*, 2006).
9. Polyclonal anti-NG2 chondroitin sulfate proteoglycan (made in rabbit; 1 : 100; Chemicon: catalogue no. AB5320, raised against purified rat NG2 chondroitin sulfate proteoglycan): this antiserum

identifies both the intact proteoglycan and the 300-kDa rat NG2 core protein as shown by immunoblotting and immunohistochemical applications (Terada *et al.*, 2006).

For counter staining, cells were incubated for 30 min with 0.001 g/mL bisbenzimidazole in 0.1 M PB (phosphate buffer) and rinsed with PBS. Finally, coverslips were mounted with a drop of 0.1 M PB.

The samples were examined with a Nikon Eclipse E800 light and epifluorescence microscope and photographed with a Nikon Coolpix 995 digital camera. Photomicrographs were manipulated and mounted in plates with Photoshop CS2 software, with autocontrast enhancement. Some preparations were examined with an Olympus Fluoview 300 confocal laser scanning microscope (CLSM).

Isolation and culture of MSCs

Seven- to nine-week-old EGFP mice were killed by cervical dislocation; their tibias and femurs were cleared of muscle and connective tissue. Bone marrow cells were aspirated using a 22-gauge needle, and washed twice for 5 min each by centrifugation at 150 g in Eagle's alpha minimum essential medium (a-MEM; Sigma) containing 2 mM L-glutamine (Invitrogen-Gibco), 100 U/mL penicillin and 100 µg/mL streptomycin (Invitrogen-Gibco).

To culture MSCs, cells were plated in 19.5-cm² polystyrene dishes (BD Biosciences) and pre-treated with a coating of fetal bovine serum (FBS; Sigma), at a density of 700 000 cells/cm²; they were grown in a-MEM supplemented with 10% FBS, in a humidified atmosphere of 95% air with 5% CO₂ at 37°C. Medium was replaced on day 4 to remove free floating cells, and then replenished every 2–3 days.

At 10 days *in vitro*, adherent cells were retrieved by trypsinisation (Trypsin, Invitrogen-Gibco) and immunodepleted of CD11b-positive granulocytic cells by magnetic cell sorting: cells were incubated with MicroBeads conjugated to monoclonal rat anti-mouse/human CD11b antibody (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) and loaded onto a MACS column (Miltenyi Biotec). Cd11b-negative cells were harvested, washed and re-plated onto dishes as described above.

When the cells were near confluence, they were washed with a-MEM and incubated with trypsin for 5 min at 37°C. Trypsin was neutralized by adding fresh complete medium. The cellular suspension was diluted 1 : 2 at each passage. Before transplantation, MSCs were trypsinized, detached from dishes, counted on a Burkert chamber as above, and re-suspended in saline solution at a final concentration of 50 × 10³ cells/µL.

Surgery and cell injection procedures

Mice were divided into three groups: (i) mice with spinal cord hemisection (HS) + saline injection (sham operated or SO mice; *n* = 6); (ii) mice with HS + transplantation of NPs (NP mice; *n* = 8); (iii) mice with HS + transplantation of MSCs (MSC mice; *n* = 6). Only animals in which the hemisection was complete and in which transplanted cells were detected at the expected neuromer are included in the current analysis and are used both for behavioural tests and for histological/immunohistochemical analysis.

Adult (2-month-old) C57BL/6J mice were deeply anaesthetized with 3% isoflurane vaporized in O₂/N₂O (50 : 50). The lower thoracic and lumbar spine was exposed and spinal muscles were displaced laterally; using a 27½-gauge needle, the left side of the spinal cord, extending from the midline through the lateral funiculus, was exposed and hemisectioned at the level of the T13 vertebra (corresponding to the L2 neuromer).

Two weeks later, under isoflurane anaesthesia (as above), a glass micropipette was used to slowly (over about 60 s) inject the

suspension of NPs or MSCs (2 µL, containing a total of 10⁵ cells in saline) into the white matter of the spinal cord at the level of the L3 neuromer; the pipette tip (outer tip diameter 50 µm) was placed 1.5 mm laterally from the midline and 1.5 mm below to the pia. SO mice received vehicle alone.

Behavioural tests

Injured mice underwent a battery of behavioural tests prior to surgery to establish a baseline for comparison with post-transplantation values; mice were tested on the following tasks before injury (day 0) and then once a week thereafter: Basso Mouse Scale (BMS), posture, grip test, foot-fault test and hindlimb flexion. The values at day 0 were obtained immediately before hemisection (representing healthy condition) and those at day 14 after SCI were acquired immediately before transplantation/vehicle administration.

Basso Mouse Scale

Hindlimb motor function was assessed in an open field, using the nine-point BMS locomotor rating scale: the gait of the mouse was observed for 4 min, categorizing hindlimb joint movements, trunk position and stability, stepping coordination, paw placement, the clearance and tail position, as described in Basso *et al.* (2006).

Posture

The mouse was placed on a smooth flat surface and its general posture was observed, from the back side. The following scores were applied: 0 = not different from normal, 1 = balance shifted to the lesioned side (adapted from Takamatsu *et al.*, 2002).

Grip test

The mouse was placed on the wire-lid of a conventional housing cage; the lid was gently shaken to prompt the mouse to hold onto the grid before the lid was swiftly turned upside down. Grip score was measured as the length of time (expressed in seconds, for a maximum of 90 s) that the mouse was able to hang on to the grid (Weydt *et al.*, 2003).

Foot-fault test

The mouse was placed on an elevated wire grid. Every time that its injured paw slipped between the wire as it moved along the grid with a weight-bearing step was recorded as a foot-fault. The total number of steps (*n* = 30) and the number of foot-faults for the injured hindlimb were counted (Pitsikas *et al.*, 2001; Zhang *et al.*, 2002).

Hindlimb flexion

The animal's head was covered with the experimenter's hand, each hindlimb was gently pulled toward the tail, and the sole of the foot was turned over. If retractive power was the same for both hindlimbs, the score was 0; if the retractive power of the left hindlimb was weaker than that of the right hindlimb, the score was 1; if the left hindlimb was extended abnormally, but was retractable when the sole was touched with a finger, the score was 2; if the left hindlimb was extended abnormally and was not retractable when the sole was touched with a finger, the score was 3 (Takamatsu *et al.*, 2002).

Histological examination

Twenty-eight days after transplantation (42 days after hemisection), animals were deeply anaesthetized by intraperitoneal injection of chloral hydrate and were perfused transcardially with buffered 4%

PFA, pH 7.4. The spinal cord was removed, cut between the T12 and L5 vertebral segments, and immersed in fixative for 2 h at 4°C. Samples were transferred overnight to PBS (pH 7.4) at 4°C, then embedded in gelatine (BDH Laboratory Supplies, Poole, UK) and cut on the vibratome (Leica model VT S1000, Microsystems, Nussloch, Germany) in the frontal longitudinal plane, at a thickness of 50 μm . Before any further processing, all sections were mounted from PBS onto a slide, coverslipped and examined with a Nikon Eclipse E800 epifluorescence microscope, using an FITC-filter; EGFP-positive transplanted cells were counted in all sections, both in the grey and white matter.

Immunofluorescence

Sections were immunostained as follows: non-specific binding sites were blocked by immersing tissue for 30 min at room temperature in 10% normal donkey serum (Sigma) in PBS, then left overnight at 4°C in primary antibody made up in the same solution. The following antibodies were used: anti-GFAP, anti-MAP-2, anti-NeuN, anti-synaptophysin and anti-serotonin (see 'Immunofluorescence of cell cultures' above). After washing in PBS, sections were incubated in cyanine-3-conjugated anti-rabbit or anti-mouse secondary antibodies (1 : 200, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) as appropriate. Sections were examined and photographed, as described above.

Immunoreactivity for 5-HT was examined to evaluate the sprouting of raphespinal axons. The white matter ipsi- and contralateral to the hemisection, was photographed at $20\times$, both about 800 μm rostral and caudal to the lesion site, using the Coolpix digital camera. For densitometric analysis, the percentage of the total area in the white matter that was 5-HT-positive was quantified using the Scion Image software for Windows (freeware version of NIH Image; Scion Corp., Frederick, MD, USA): the background was subtracted from photographs by manual thresholding by two blinded observers.

Survival of transplanted cells

Transplanted cells were counted in the spinal cord to quantify the number that had survived for 28 days following transplantation. EGFP⁺-NPs or MSCs were counted in all sections: in order to avoid double counting in consecutive sections, only cells contained entirely within the section were counted; partial cells, cut at the edges of the section, were not included in the counts.

Nissl staining

Several sections from each animal were stained for Nissl substance to identify the extent of the hemisection and to characterize the histology of the lesion site and of the uninjured host spinal cord. Sections were mounted on 2% gelatin-coated superfrost plus slides and air-dried overnight; slides were hydrated in distilled water for 1 min before placing in 0.5% Cresyl violet acetate for 6 min, dehydrated in an ascending series of ethanol, cleared in xylene and cover-slipped with Eukitt (Biotica, Milan, Italy).

Statistics

Data are shown as mean \pm SEM (standard error of the mean), and inter-group differences were compared statistically with one-way ANOVA and two-tailed paired Student's *t*-test. Differences were considered significant at $P \leq 0.05$.

Results

Site of damage

Evidence of degeneration was visible at the lesion site; in fact, the Nissl-stained sections revealed a dramatically altered morphology of the spinal cord. Numerous glial cells and apoptotic profiles (Fig. 1a) were present both at and around the injury site (inset of Fig. 1a), in the white matter as well as the grey matter. Cystic cavity due to the needle, also seen in GFAP-immunostained sections (Fig. 1b), was visible; hypertrophic astrocytes defined the glial scar and delineated the border of the lesion. Within the cyst, cellular density was very high due to the presence of glial progenitors, microglia, macrophages and fibroblasts (Thuret *et al.*, 2006), but the classical star-shaped GFAP-positive cells were not detectable. Tissue surrounding the lesion site was also

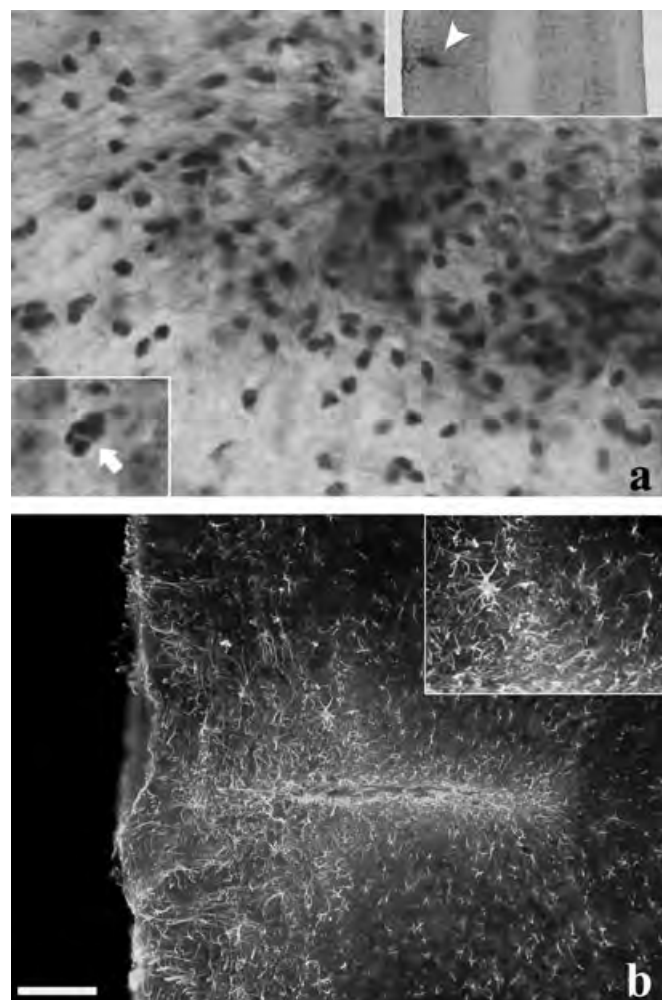


FIG. 1. Hemisection site. (a) Nissl staining of a frontal longitudinal section, in the lesion area (arrowhead in the upper inset, scale bar = 900 μm). The lesion is characterized by intense staining around and inside the injury site, into the white and grey matter, showing a massive increase in glial cells and marked apoptosis of neural cells (apoptotic bodies of a pyknotic nucleus in the lower inset, scale bar = 7 μm). (b) GFAP immunofluorescence of a frontal longitudinal section. The glial scar is characterized by hypertrophic astrocytes, delineating the border of the lesion. The surrounding tissue is also occupied by star-shaped astrocytes (at a higher magnification in the inset, scale bar = 100 μm), but at a lower cellular density than in proximity of the lesion site. By contrast, the narrow glial cyst is completely empty of astrocytes. Scale bars = (a) 20 μm (b) 150 μm .

characterized by hypertrophic astrocytes, but at a lower density than in the proximity of the lesion site (inset of Fig. 1b).

Differentiation of neural precursors *in vitro*

Some neural precursors, obtained from neural tube of EGFP⁺ embryos, were cultured for 3 days in the complete medium previously described by Mitsui *et al.* (2005), to maintain a mixed cellular population. During the culturing period, the cells showed a polymorphic aspect, from round to elongated, frequently displaying numerous long processes (Fig. 2a and b).

NP cultures mainly expressed the early neural marker, nestin (64% of plated cells) (Fig. 2c–e). Cultured NPs were negative to NeuN, MAP-2, 5-HT, synaptophysin, GFAP, vimentin, NG2 and MBP, thus suggesting an undifferentiated state of cultured precursors.

Differential survival and distribution of NPs vs. MSCs

Hemisection was performed at vertebral level T13 (left side) and, 2 weeks later, NPs or MSCs were injected ipsilaterally into the severed spinal cord, one neuromer below the injury.

Animals were killed 28 days after transplantation (42 days after hemisection). Comparisons made across animals at this time point

revealed that many more NPs had survived relative to MSCs: $1.96\% \pm 1.18$ of the transplanted NPs were visible vs. $0.19\% \pm 0.10$ of the transplanted MSCs. However, no masses of EGFP-positive cells were visible infiltrating the surrounding tissue and blood vessels, thus excluding tumour formation.

NPs were detected in clusters close to the injection site (Fig. 3a), but they had also migrated rostrally from the L3 neuromer towards the hemisection site (at the L2 neuromer). Generally they stopped just short of the lesion site, but in one animal a central stream of grafted cells extended around and up to $500\ \mu\text{m}$ cranial to the injury site (Fig. 3b). Other streams extended up to $1200\ \mu\text{m}$ caudal to the graft site, and $300\ \mu\text{m}$ medial to it, towards the central canal. Some of these cells had very long axons and collateral branches with arbors (Fig. 3c).

MSCs, by contrast, did not migrate far: most remained in clusters (Fig. 3d–g), close to the injection site. Rarely, cells were visible about $150\ \mu\text{m}$ caudal and $125\ \mu\text{m}$ medial (i.e. towards the grey matter) to the injection site. In one animal, a small stream of EGFP-positive MSCs was seen one level rostral to the hemisection (data not shown).

Differentiation of transplanted NPs and MSCs

One month after transplantation, the morphology of NPs was extremely heterogeneous: some cells displayed a fibroblast-like shape,

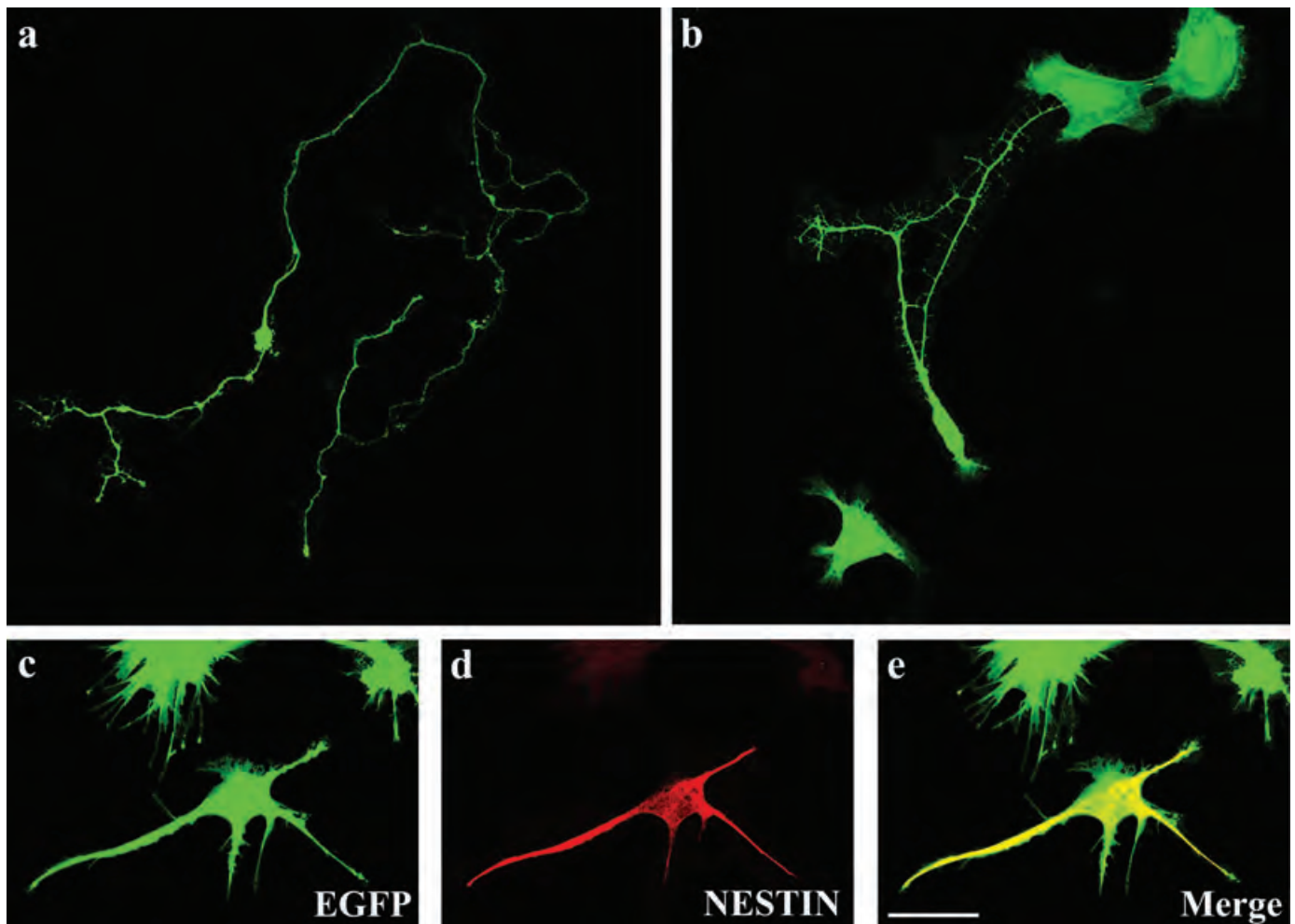


FIG. 2. Differentiation of neural precursors *in vitro*. (a, b) *In vitro* NPs showing a polymorphic aspect, from round to elongated, frequently showing long processes. (c–e) Differentiation of cultured NPs is assessed by co-localization of EGFP (green) and marker against nestin (labelled in red) in the CLSM. Scale bar = $30\ \mu\text{m}$.

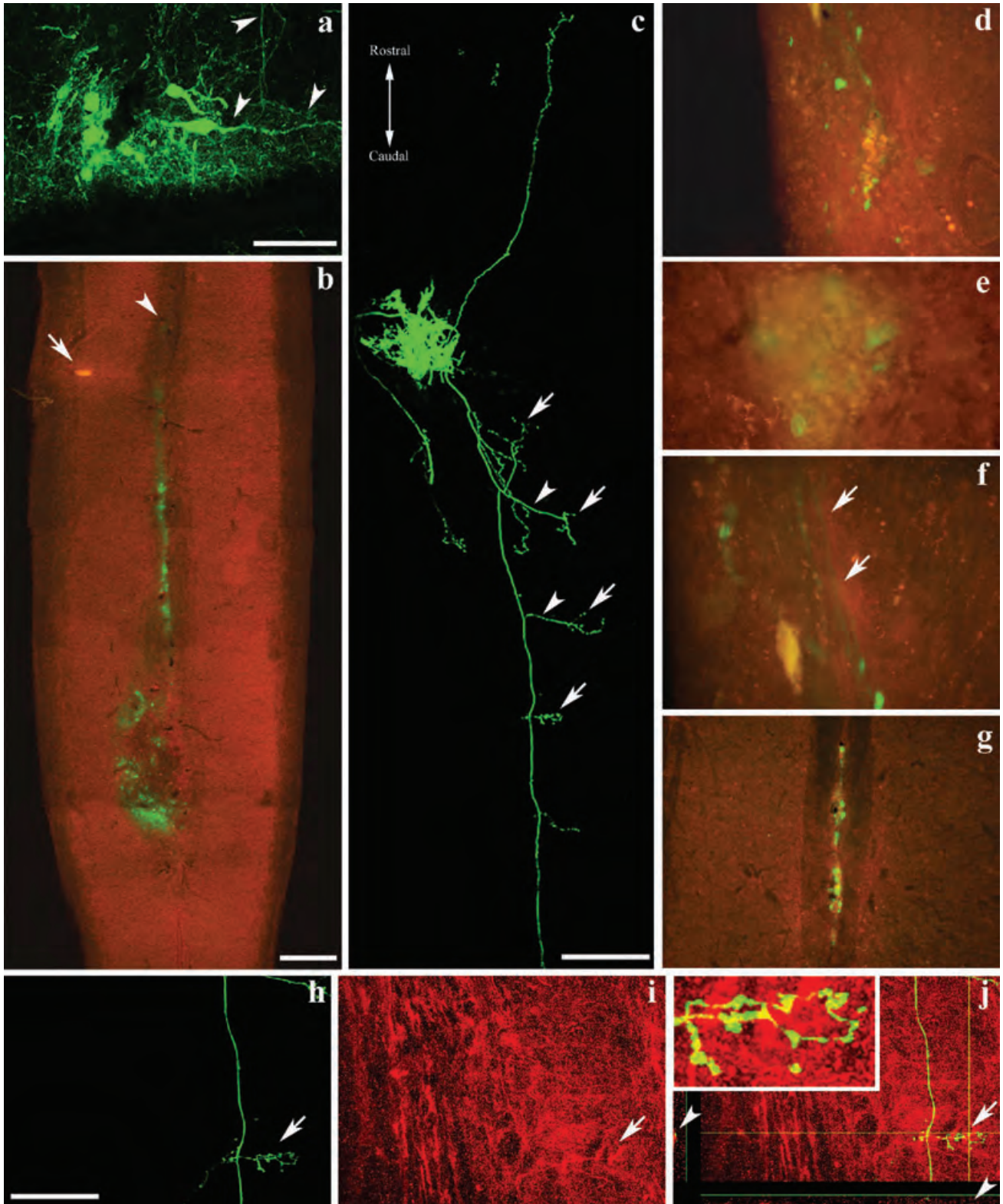


FIG. 3. Survival and distribution of NPs and MSCs. (a) NPs display heterogeneous morphology, from fibroblastoid-like shape to neuron-like phenotype characterized by more or less elongated processes (arrowheads). NPs are distributed in clusters close to the injection site, one neuromer below the injury site. (b) Even though NPs migrate rostrally in the white matter but stop before arriving to the lesion site, in one animal a central bundle of grafted cells extended around 500 μm cranially (arrowhead) to the injury site (arrow). (c) NPs in the lateral funiculus of hemisectioned mice, characterized by an elongated axon bearing several collaterals (arrowheads) and synaptic boutons (arrows). (d) MSCs with a fibroblastoid-like shape, (e) organized in clusters close to the injection site, (f) rarely in small bundles (arrows). (g) Small bundle of EGFP⁺ MSCs. (h) Transplanted NPs show synaptic boutons (at higher magnification in the inset) that are in contact (arrows) with local neurons (j), expressing 5-HT marker (i). In (j) rotations along the *x*- and *y*-axes show the superposition of the two colours on the *z*-axis (arrowheads). Scale bar = 100 μm in a, c, d and g, 50 μm in e and f, 500 μm in b and 80 μm in h–j (17 μm for the inset).

others exhibited a neuronal phenotype characterized by more or less elongated processes (as shown for the cluster of neurons in Fig. 3c). EGFP-positive NPs in the cord were positive for 5-HT (8.48%), MAP-2 (18.8%) or NeuN (54%) (Fig. 4a–i), but were negative for the astrocyte marker GFAP. In the lateral funiculus of three hemisectioned mice, we observed NPs characterized by an elongated axon in contact with local neurons (Fig. 3h–j), with several collaterals bearing bouton-

like swellings and arborizing around local cells (Fig. 3c) (Tettoni *et al.*, 1998). Many of these EGFP-positive cells expressed synaptophysin (Fig. 4j–l). One month following SC transplantation, such axons could be followed up to 400 μm cranially and 700 μm caudally from the injection site (Fig. 3c).

MSCs retained a fibroblast-like, fusiform shape. Rarely, MSCs (similar to bipolar neurons) emitted processes that extended cranially;

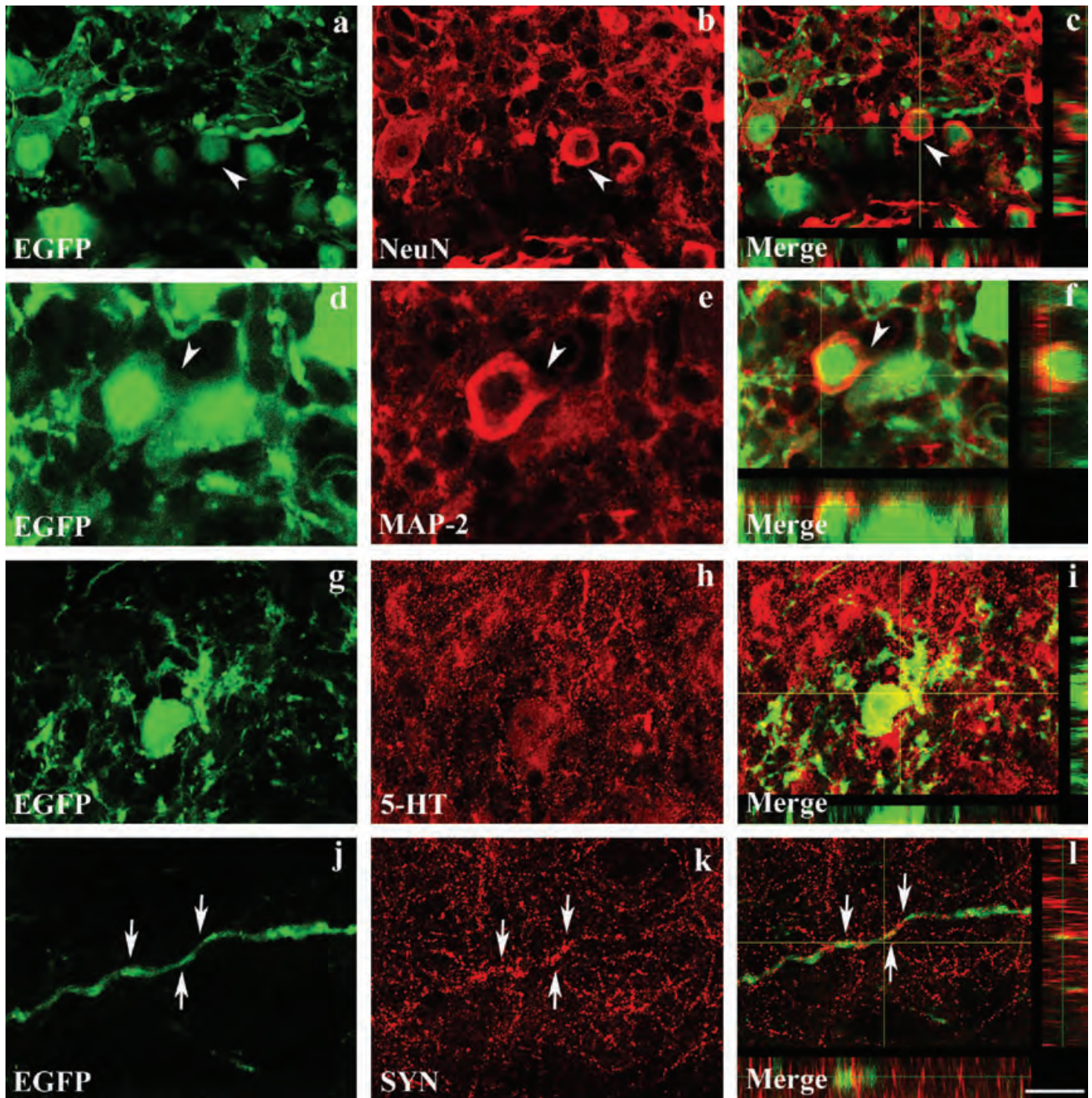


FIG. 4. Co-localization of NPs with neural markers. Differentiation of NPs within host spinal cord, 28 days after transplantation, is assessed by co-localization of EGFP (green) and neuronal markers (labelled in red) at the CLSM. (a–c) Immunoreactivity against NeuN (arrowhead). (d–f) Immunoreactivity against MAP-2 (arrowhead). (g–i) Immunoreactivity against 5-HT. (j–l) Immunoreactivity against synaptophysin (SYN) along a EGFP⁺ axon (arrows). In c, f, i and l, rotations along the x- and y-axes show the superposition of the two colours on the z-axis. Scale bar = 20 μm in a–c and g–i, 10 μm in d–f and j–l.

these were, at most, about 200 μm in length (Fig. 3d–g). In contrast to the transplanted NPs, no engrafted MSCs expressed neuronal or glial markers.

Sprouting of 5-HT-positive axons induced by engrafted NPs and MSCs

The density of 5-HT-positive fibres was quantified in the lateral funiculus of the three experimental groups, at the L1–L2 neuromer and the L2–L3 neuromer, cranial and caudal to the site of the lesion, both ipsilateral and contralateral to the hemisection (Fig. 5). Fibre density was expressed as the percentage of coverage of 5-HT-positive profiles in photographs of longitudinal sections through the lateral funiculus. Data are presented in Table 1.

In summary, 42 days after hemisection, a 39.62% reduction of immunopositive profiles was observed on the side of the lesion compared with fibre density rostral to SCI, whereas the numbers were essentially unchanged on the opposite side. Following engraftment of NPs and MSCs, the density of serotonergic ipsilateral fibres caudal to the site of hemisection observed was increased by 22.27 and 21.84%, respectively, over that in SO mice and these results were statistically significant (ANOVA, $P < 0.01$ and $P < 0.05$). Finally, serotonergic axon bundles were close to clusters of grafted NPs, suggesting that the cells might contribute to the sprouting or sparing of 5-HT projections (Fig. 3c and h–j).

Behavioural outcome

All behavioural tests were performed by two researchers (M.B. and R.R.) who were ‘blinded’ as to the group to which the mouse belonged. Recovery of motor behaviour in treated vs. untreated SCI mice was evaluated with a battery of tests: BMS, posture, grip test, foot-fault test and hindlimb flexion (Fig. 6). Scores reported were obtained before hemisection (day 0) and then every week until the animals were killed (day 42): results on the 14th day post-injury were obtained just prior to the injection of the SCs/saline.

The 2-week interval prior to the engraftment allowed mice to recover from the acute phase of hemisection. The performance in single tests at 1–2 weeks from lesion (i.e. before transplantation or saline injection) was variable among the different experimental groups, even though none of the groups behaved worse than all the others. Nevertheless, these differences were not statistically significant. On the contrary, when differences between performances at day 42 and day 14 were compared between transplanted and SO mice, most tests gave significant positive outcome for cell-transplanted mice.

Basso Mouse Scale

On the 42nd day post-injury, SO mice showed no coordination between forelimbs and hindlimbs and occasional plantar stepping of the injured (left) hindlimb (BMS score = 4.67 ± 0.73). Four weeks after transplantation, NP mice improved coordination and showed frequent or consistent plantar stepping (BMS score = 5.38 ± 0.78). MSC mice also improved similarly, showing also the paw digits parallel to the body at initial contact during locomotion (BMS score = 6.17 ± 0.52). In summary, compared with performance at day 14, we documented respectively a 2.29-point improvement in the BMS score of NP mice (ANOVA $P < 0.05$) and a 3-point improvement in that of MSC mice (ANOVA $P < 0.01$), whereas SO mice performance was almost unchanged (0.17-point improvement).

Posture

With this test we evaluated general balance. Data obtained were in accordance with BMS test records. At the 21st day after hemisection, all mice showed a similar performance (about 0.75 points), with posture shifted towards the injured left side. In the ensuing weeks, both NP and MSC mice had gradually improved to 0.38 ± 0.07 and 0.33 ± 0.06 points, respectively, at 6 weeks post-transplantation. In contrast, after an initial improvement (better performance at day 28 with 0.53 ± 0.10), SO mice worsened progressively, displaying at day 42 a score of 0.67 ± 0.06 . Therefore, NP, MSC and SO mice showed a performance improvement of about 50, 67 and 17%, respectively (t -test SO vs. MSC $P < 0.05$).

Foot-fault test

Following hemisection, the performance of NP and MSC mice improved steadily, until day 42 when NP animals performed 4.21 ± 0.98 foot-faults and MSC animals 3.06 ± 0.41 . By contrast, on the day the animals were killed, the SO group registered 8.28 ± 1.42 foot-faults. Therefore, between the 14th and the 42nd day NP mice improved by 11.9%, MSC by 12.79% and SO worsened by 0.74%.

Hindlimb flexion

This test evaluates the retractile power of hindlimbs, comparing the left injured side with the undamaged side. Four weeks post-transplantation, we documented a significant improvement in both the NP and the MSC groups: the score displayed by NP mice was 0.67 ± 0.24 and that shown by MSC mice was 0.33 ± 0.14 . SO mice displayed a similar final score (0.72 ± 0.23), but relative to the performance 2 weeks following lesion NP mice improved by 42.87% (t -test $P < 0.05$), MSC by 51.87% (ANOVA $P < 0.05$) and SO only by 9.27%.

Grip test

Animals were evaluated with the grip test to examine the resistance force of the damaged hindlimb. Forty-two days after hemisection, both transplanted groups displayed a positive performance outcome, resisting almost 90 s as required from the test. SO performance showed a relative improvement between days 14 and 42 by 0.18%. By contrast, NP mice improved by 7.17% and MSC mice by 10.43%.

Discussion

We have compared the survival and integration of NPs or MSCs transplanted into the mouse spinal cord following hemisection; we studied the effects of the transplantation on the sprouting of raphespinal axons. We show that both cell types are able to survive for a long time and in fact they may have integrated into neural circuits of the injured spinal cord; both types promote sprouting of raphespinal axons under the lesion and functional recovery of motor behaviour.

Methodological issues

The success of cell transplantation is critically dependent on the timing of the cell injection (Pearse & Bunge, 2006) relative to the development of inhibitory influences such as neurotoxicity caused by inflammatory cytokines in the acute phase, and an enlarging cystic cavity, which prevent axonal regeneration in the chronic phase (Okano, 2002). Nonetheless, if the grafting neural stem/progenitor

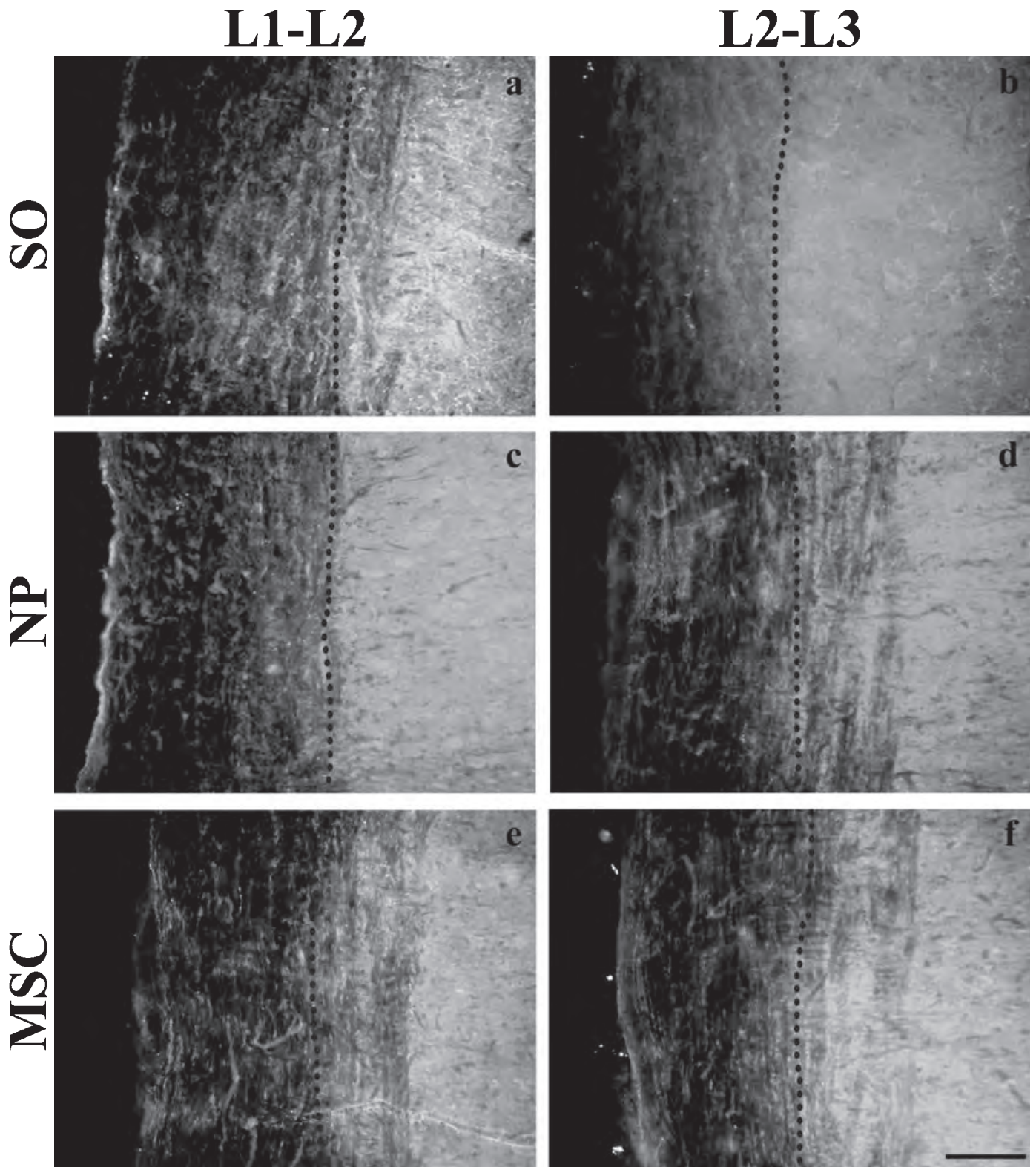


FIG. 5. 5-HT immunoreactivity. 5-HT-positive fibres in the spinal cord of SO (a and b), NP (c and d) and MSC (e and f) mice, cranially (a, c, e) and caudally (b, d, f) to the hemisection. In SO mice there is an evident decrease in 5-HT-positive fibres caudally to the lesion, not so remarkable in transplanted mice. 5-HT-positive fibre density in the white matter at spinal cord levels cranially (L1–L2 neuromer level) and caudally (L2–L3 neuromer level) to the lesion site was assessed by the Scion Image software. Broken lines border the area of interest of the white matter used for quantification. Scale bar = 100 μ m.

TABLE 1. Quantitative analysis of 5-HT-positive fibres 42 days after hemisection in the lateral funiculus of neural precursor (NP), mesenchymal stem cell (MSC) and sham-operated (SO) mice

Spinal level	SO	NP	MSC
L1–L2 left	23.07 ± 1.35	21.52 ± 1.03	22.30 ± 0.67
L2–L3 left	13.76 ± 0.99	16.73 ± 1.29	17.88 ± 1.02
L1–L2 right	20.65 ± 1.00	21.02 ± 0.49	20.42 ± 0.58
L2–L3 right	19.74 ± 0.79	19.51 ± 0.75	20.71 ± 1.08

Data are reported as mean ± SEM. SO mice display a 39.62% reduction of immunopositive profiles relative to the density of ipsilateral fibres cranial to the lesion, whereas NP and MSC mice display only 22.27 and 21.84%, respectively (ANOVA, $P < 0.01$ and $P < 0.05$, respectively). The table summarizes data relative to fibre quantification.

cells following spinal contusion is delayed, the cells survive for a long time and lead to a long-lasting functional recovery (Okada *et al.*, 2005). We thus chose to transplant NPs and MSCs 2 weeks after hemisection.

Cells were injected into the neuromer just caudal to the hemisection, in an intact host environment where they might survive more readily. Forty-two days after hemisection and 28 days after engraftment of 10^5 stem cells, 1.96% NPs and 0.19% MSCs survived. This is probably an underestimate, due to the section thickness, the presence of cell clusters in which individual profiles were difficult to resolve and the long post-transplantation time interval. In an earlier study, we have shown that transplantation of similar numbers of MSCs in a mouse model of amyotrophic lateral sclerosis delays the progression of the disease (Vercelli *et al.*, 2008). In the current study, even though cell survival appears to be comparable with that reported for other studies (Hofstetter *et al.*, 2002; Wu *et al.*, 2003), the relatively greater numbers of surviving NPs may be due to their origin in the embryonic spinal cord, as opposed to the MSCs, which are harvested from the bone marrow of the tibia and femur, and are transplanted in an environment that is completely different from where they would normally reside. Moreover, the expansion of MSCs for least 2 weeks prior to transplantation could represent a stress factor affecting survival.

Survival and differentiation of transplanted cells

NPs seem to be attracted to the site of hemisection, as indicated by their preferential rostral migration; their elongated cell bodies are aligned along the direction of migration, as also shown by Han *et al.* (2004): such tropism may be mediated by chemotactic factors such as chemokines, proinflammatory cytokines (TNF α) and growth factors (FGF2 and PDGF) (Ben-Hur *et al.*, 2003). Thus, the presence of myelinated axons might not be permissive for axon growth, but these axons may allow migration of precursor cells (Han *et al.*, 2004), which could participate in new spinal circuits and make the environment permissive for axonal growth at the injury site, as suggested by Lepore & Fischer (2005).

With the paradigm used for this study, the NPs express a number of neuronal (but no glial) markers and elaborate long axon-like processes with several collaterals. The bouton-like swellings and also the synaptophysin immunoreactivity associated with the grafted cells suggest that they make, as well as receive, synaptic boutons (Tettoni *et al.*, 1998). The failure of a glial phenotype seemingly contradicts other reports, in which NPs take on phenotypes of astrocytes as well as of oligodendrocytes when injected into intact spinal cord, or into many models of contusion or compression injury. Thus, NPs have the

capacity to integrate in the host, even in a non-permissive environment (Cao *et al.*, 2002; Han *et al.*, 2002, 2004; Lepore & Fischer, 2005; Lepore *et al.*, 2005; Mitsui *et al.*, 2005; Karimi-Abdolrezaee *et al.*, 2006).

NPs give rise to glial restricted precursors (GRPs), and express GFAP only when they become 'astrocyte precursor cells' (Rao, 1999): therefore, environmental factors could inhibit GRP differentiation into mature astrocytes when transplanted into the injured spinal cord in proximity of lesion site (as in our work) and not directly inside the lesion area (as in other studies such as Cao *et al.*, 2002; Han *et al.*, 2004; Lepore & Fischer, 2005; Mitsui *et al.*, 2005; Karimi-Abdolrezaee *et al.*, 2006). In addition, we show that *in vitro* NPs are positive for nestin but not for other mature neuronal or glial markers, suggesting that they remain undifferentiated immediately after collection and mature in the injured tissue.

MSCs in our study were negative for both neuronal and glial markers. Many authors have reported on the ability of MSCs to differentiate into neurons and glia following central nervous system (CNS) injury (Phinney & Isakova, 2005; Thuret *et al.*, 2006; Coutts & Keirstead, 2008), even though the evidence indicates that this results from a process of cell fusion rather than that of transdifferentiation (Terada *et al.*, 2002; Wurmser & Gage, 2002). Also, most studies that report on the differentiation of MSCs into neurons have used only immunolabelling and morphological criteria, with no electrophysiological data to demonstrate neuronal properties (Lu & Tuszynski, 2005). In the present study, MSCs do not transdifferentiate into neurons, nor into glia, but they nonetheless do contribute to functional recovery, presumably via a neurotrophic role, as they reportedly secrete trophic and growth factors, such as BDNF, VEGF, NGF and NT-3, constitutively (Lu *et al.*, 2005; Neuhuber *et al.*, 2005; Mareschi *et al.*, 2006).

Axonal growth

Serotonergic neurotransmission regulates the motor, somatosensory and limbic systems (Hornung, 2003) and their re-growth therefore has important functional implications. Descending serotonergic fibres promote functional recovery after SCI (Faden *et al.*, 1988; Hashimoto & Fukuda, 1991; Schmidt & Jordan, 2000). In the present study, serotonergic fibres ipsilateral and caudal to the hemisection were cut. Spontaneous axonal repair after SCI has been reported in animals and humans (Fawcett & Asher, 1999; Bareyre *et al.*, 2004), especially for serotonergic fibres (Schmidt & Jordan, 2000). We were interested in enhancing this spontaneous regrowth with the use of SCs. Spinal cord hemisections are usually not complete, and in our model, many contralateral fibres can be detected, crossing the midline caudal to the injury. Eidelberg *et al.* (1981) report that even a 25% sparing of descending fibres is sufficient for coordinated hindlimb locomotion in monkeys. Following unilateral spinal cord lesions in the developing animal, corticospinal fibres from the undamaged side sprout towards the denervated region, and are able to mediate behavioural recovery (Rouiller *et al.*, 1991), whereas such sprouting is more limited when lesions are made in adulthood (Aoki *et al.*, 1986; Goldstein *et al.*, 1997), but still considerable (Bareyre *et al.*, 2004; Soares *et al.*, 2007). New functional intraspinal circuits may also develop in response to injury: transected hindlimb corticospinal axons sprout into the cervical grey matter where they contact and stabilize synapses on propriospinal neurons that were spared by the lesion; these neurons in turn increase their input on lumbar motoneurons, thus creating new intraspinal circuits, which correlates improvement of hindlimb function (Bareyre *et al.*, 2004).

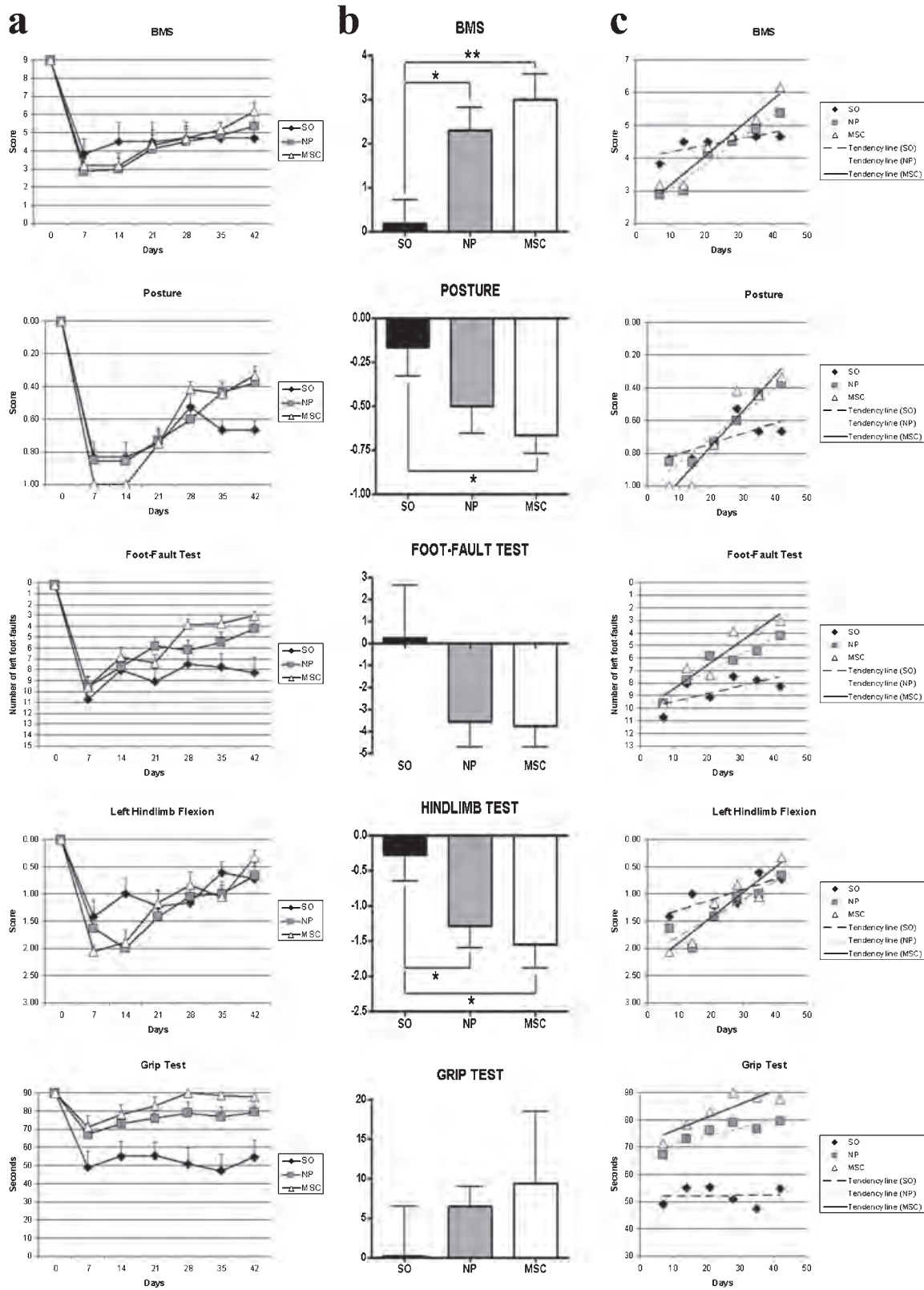


FIG. 6. Behavioural tests. Analysis of functional recovery of NP (filled grey square), MSC (empty triangle) and SO (filled black diamond) mice, studied with a battery of tests (BMS, posture, foot-fault test, hindlimb flexion, grip test). Column (a) shows the scores obtained immediately before hemisection (day 0) and then once 1 week thereafter (those at day 14 after SCI were acquired immediately before transplantation/vehicle administration). The scores are expressed as mean \pm SEM. Column (b) displays the differences in SO, NP and MSC performances between days 42 and 14: positive values in the BMS and grip test and negative values for the other tests reflect improvements in the performances ($*P < 0.05$; $**P < 0.01$). In column (c) the same data as in column (a) are shown as a tendency line (without SEM), in order to put in evidence the trend to improvement.

The persistence of 5-HT-positive fibres in the spinal cord caudal to the hemisection in SO mice is similar to earlier reports on spontaneous recovery of serotonergic fibres following complete cord transection (Hadjiconstantinou *et al.*, 1984). However, we observed 40% reduction in fibre density on the hemisectioned side caudal to the lesion. The increased number of serotonergic axons that we see in response to transplantation of NPs and MSCs is similar to the observations of Mitsui *et al.* (2005) for a contusion model, and leads to the conclusion that the grafted precursors promote sprouting of serotonergic fibres from the contralateral side (Hill *et al.*, 2004; Pfeifer *et al.*, 2004): in fact, we did not observe 5-HT axons crossing the damaged area, even though a number of studies have reported ability of other types of SCs to promote regeneration of severed ipsilateral serotonergic axons following SCI (Lu *et al.*, 2002; López-Vales *et al.*, 2007). Many authors in fact have hypothesized that SCs are effective in optimizing the plastic response of severed fibres, both serotonergic and otherwise, and in synaptic remodelling (Harel & Strittmatter, 2006).

MSCs also promote the sparing or sprouting of 5-HT-positive injured axons. In the contused spinal cord, MSCs form bundles that bridge the epicentre of the lesion or are located close to 5-HT-positive fibres (Hofstetter *et al.*, 2002). Their secretion of neurotrophic factors can enhance sensory and motor axon regrowth (Lu *et al.*, 2005; Neuhuber *et al.*, 2005). Crigler *et al.* (2006) demonstrated that neurotrophin expression levels in human MSCs are correlated with the ability of these cells to promote survival and induce neurite formation in neuroblastoma cells and cultured peripheral neurons. Neurotrophin secretion by MSCs could be further enhanced by the lesioned environment in the hemisectioned spinal cord (Neuhuber *et al.*, 2005).

Serotonergic fibres could integrate into spinal circuits after hemisection either following stimulation of local guidance cues expressed by the lesioned spinal cord, or by following activity-dependent pruning of inappropriate connections to refine and consolidate newly formed synapses. Denervation of the spinal cord leads to a recapitulation of development in that expression of trophic factors, axonal guidance molecules and extracellular matrix proteins is enhanced (Maier & Schwab, 2006). In addition, NPs and/or MSCs produce immunomodulatory molecules which prevent astrogliosis and microglia activation, thus creating a permissive environment to fibre sprouting.

Moreover, we found 8.48% NPs positive for 5-HT, suggesting that they might participate in the reorganization of serotonergic fibres as well, which could, in part, explain the higher density of serotonergic fibres caudal to the hemisection in transplanted animals compared with SO animals.

Our findings and those of other studies using SC transplantation in SCI models indicate that serotonergic fibres may be less affected by inhibitory factors such as Nogo relative to other axons (Fawcett & Asher, 1999; Schwab, 2004). When the glial scar is minimized, axons can grow considerable distances in the white matter (Davies *et al.*, 1997), and sufficient amounts of growth-promoting factors at the injury site can overcome the inhibitory signals (Jones *et al.*, 2003). Also, growing fibres can enter the scar at the lesion site if bone marrow stromal cells that have been genetically modified to secrete NT-3 are injected at the site of damage (Lu *et al.*, 2007).

Functional outcome

Our results show that SC transplantation near the lesion site significantly improves functional recovery in animals with SCI: in fact, all tests – from simple observation of posture to evaluation of a

resistance force and hindlimb coordination – displayed significant improvements in grafted groups compared with SO mice. In contrast, the performance of SO mice on day 42 following surgery was the same or only slightly improved than that at day 7.

Although aberrant axonal allodynia-like hypersensitivity of forepaws has been reported following graft (Hofstetter *et al.*, 2005), our sensory tests (data not shown) revealed only a slight hypersensitivity in rare cases during the 6 weeks of observations, both in transplanted and in SO mice.

The involvement of 5-HT in regulating motor activity (Hornung, 2003) and the higher density of 5-HT-positive fibres observed in our transplanted groups, relative to SO mice, can explain the functional recovery noted in the battery of motor behaviour tests used to evaluate mice. Dense serotonergic innervation of the lumbar spinal cord in normal animals is believed to control hindlimb locomotion (Kiehn, 2006).

Simple locomotor behaviours such as walking do not depend on the corticospinal tract (Metz *et al.*, 1998); instead, the phylogenetically older rubro-, vestibulo- and reticulospinal tracts, the monoaminergic systems originating in the brainstem, and intrinsic spinal circuits mediate such behaviours; recovery of function on locomotor tasks may be due to synaptic plasticity in pre-existing circuits, anatomical and functional reorganization, and formation of new circuits (Raineau & Schwab, 2001). Each of the projections displays different levels of sprouting following SCI. Serotonergic fibres originate from the raphe nuclei (Dahlstrom & Fuxe, 1964) and project to the ventral horn, where they contact motoneurons and interneurons (Bowker *et al.*, 1982). Blockade of the neurite growth inhibitor Nogo-A restores the descending serotonergic raphespinal tracts and promotes functional recovery (Müllner *et al.*, 2008) and direct transplantation of embryonic serotonergic cells has been shown to improve motor functions (Feraboli-Lohnherr *et al.*, 1997).

Conclusions

Our results support a positive effect of SC transplantation with regard to anatomical and functional recovery following SCI, both with use of embryonic neural cells and adult MSCs. NPs and MSCs survive many weeks in the absence of immunosuppression, integrate into the injured host spinal cord and its circuits, and promote functional recovery after hemisection. These SCs are thus strong candidates for cell transplantation therapy following SCI. In accordance with other studies, both cell types could act as biological minipumps that migrate towards the lesion site and deliver trophic factors and immunomodulatory molecules. Their therapeutic potential could be further exploited by inserting genes coding for specific neurotrophic factors or immunomodulatory molecules. MSCs have distinct advantages in that they can be collected from each patient, by-passing ethical concerns about use of embryos to harvest SCs, and can be expanded *in vitro*. MSCs have also been used for many decades for bone marrow transplantation, thus reducing concerns about their safety. Nevertheless, further studies should explore a combination therapy using both cell types, which could have additive positive results.

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Abbreviations

5-HT, serotonin; a-MEM, Eagle's alpha minimum essential medium; BMS, Basso Mouse Scale; BSA, bovine serum albumin; CNS, central nervous system; CLSM, confocal laser scanning microscope; EGFP, enhanced green fluorescent protein; FBS, fetal bovine serum; GFAP, glial fibrillary acidic protein; GRPs, glial restricted precursors; HS, hemisection; MAP-2, microtubule-associated protein-2; MBP, myelin basic protein; MSCs, mesenchymal stem cells; NeuN, neuronal nuclei; NPs, neural precursors; NT-3, neurotrophin-3; PFA, paraformaldehyde; PB, phosphate buffer; PBS, phosphate-buffered saline; SC, stem cell; SCI, spinal cord injury; SEM, standard error of the mean; SO, sham operated; SYN, synaptophysin.

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