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NEUROSYSTEMS

Undesired effects of a combinatorial treatment for spinal cord injury – transplantation of olfactory ensheathing cells and BDNF infusion to the red nucleus

Frederic Bretzner, Jie Liu, Erin Currie, A. Jane Roskams and Wolfram Tetzlaff
ICORD (International Collaboration On Repair Discoveries), Departments of Zoology and Surgery, University of British Columbia, Vancouver, BC V6T 1Z4, Canada

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Abstract

Transplantations of olfactory ensheathing cells (OECs) have been reported to promote axonal regeneration and functional recovery after spinal cord injury, but have demonstrated limited growth promotion of rat rubrospinal axons after a cervical dorsolateral funiculus crush. Rubrospinal neurons undergo massive atrophy after cervical axotomy and show only transient expression of regeneration-associated genes. Cell body treatment with brain-derived neurotrophic factor (BDNF) prevents this atrophy, stimulates regeneration-associated gene expression and promotes regeneration of rubrospinal axons into peripheral nerve transplants. Here, we hypothesized that the failure of rubrospinal axons to regenerate through a bridge of OEC transplants was due to this weak intrinsic cell body response. Hence, we combined BDNF treatment of rubrospinal neurons with transplantation of highly enriched OECs derived from the nasal mucosa and assessed axonal regeneration as well as behavioral changes after a cervical dorsolateral funiculus crush. Each treatment alone as well as their combination prevented the dieback of the rubrospinal axons, but none of them promoted rubrospinal regeneration beyond the lesion/transplantation site. Motor performance in a food-pellet reaching test and forelimb usage during vertical exploration (cylinder test) were more impaired after combining transplantation of OECs with BDNF treatment. This impaired motor performance correlated with lowered sensory thresholds in animals receiving the combinatorial therapy – which were not seen with each treatment alone. Only this combinatorial treatment group showed enhanced sprouting of calcitonin gene-related peptide-positive axons rostral to the lesion site. Hence, some combinatorial treatments, such as OECs with BDNF, may have undesired effects in the injured spinal cord.

Introduction

Olfactory ensheathing cells (OECs) derived from the olfactory bulb (OB) have been reported to promote axonal regeneration, remyelination, as well as functional recovery after various types of spinal cord injury (Li *et al.*, 1997; Ramon-Cueto *et al.*, 1998, 2000; Nash *et al.*, 2002; Keyvan-Fouladi *et al.*, 2003; Sasaki *et al.*, 2004, 2006a). Claims of the clinical benefits of OECs in spinal cord injury are being made in several clinical centers (Huang *et al.*, 2003; Amador & Guest, 2005); however, these treatments lack rigorous controls and characterization of the actual cells transplanted (Dobkin *et al.*, 2006; Guest *et al.*, 2006). Because of its greater accessibility the nasal mucosa is the favored source of OECs for autologous transplantation in humans (Lu *et al.*, 2001, 2002). We have previously shown that lamina-propria (LP)-derived OECs from the mouse nasal mucosa share several similar properties to those derived from the OB *in vitro* (Au & Roskams, 2003) as well as *in vivo* (Ramer *et al.*, 2004a; Richter *et al.*, 2005), but do demonstrate some differences before and after transplantation (Richter *et al.*, 2005). However, only a few rat rubrospinal axons regenerated into engrafted mouse LP-OECs but not beyond (Ramer *et al.*, 2004a), and similarly into rat OB-OECs overexpressing brain-derived neurotrophic factor (BDNF; Ruitenberg *et al.*, 2003). This inability of rubrospinal axons to regenerate might be due, in part, to a weakness of the intrinsic cell body response of the red nucleus (Tetzlaff *et al.*, 1991; Jenkins *et al.*, 1993). We have previously shown that cell body treatment of the red nucleus with BDNF prevented the atrophy of rubrospinal neurons, stimulated the expression of regeneration-associated genes and promoted axonal regeneration of the rubrospinal axons into free-ending peripheral nerve transplants grafted into the cervical spinal cord injury site (Kobayashi *et al.*, 1997). This strategy was still successful even when initiated 1 year after spinal cord injury (Kwon *et al.*, 2002).

We therefore hypothesized that a BDNF-induced enhancement in the rubrospinal cell body response would promote regeneration of rubrospinal axons into and beyond a LP-OEC transplant, and would enhance functional recovery of the forelimb following a cervical lesion of the spinal cord. To assess this, we combined a transplant of highly enriched preparations of LP-OECs derived from the nasal mucosa of green fluorescent protein (GFP)-expressing neonatal mice

Correspondence: Dr W. Tetzlaff, as above.
E-mail: tetzlaff@icord.org

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with a cell body treatment of the red nucleus delivering BDNF for 2 weeks. This combinatorial approach enhanced some regenerative sprouting of rubrospinal axons but failed to promote their regeneration through engrafted LP-OECs. Unexpectedly, this combined strategy diminished and delayed the functional recovery of the injured forelimb, which correlated with decreased sensory thresholds.

Materials and methods

Preparation of LP-OECs from GFP transgenic mice

The preparation of OECs is detailed elsewhere (Ramer *et al.*, 2004a). In brief, OECs were harvested from the olfactory mucosa of postnatal day 5 transgenic mice (killed by decapitation) expressing GFP under the beta-actin promoter (Au & Roskams, 2003; Ramer *et al.*, 2004a; Richter *et al.*, 2005). The entire olfactory mucosa, including turbinates and septum, was dissected from one pup, mechanically dissociated and treated with 0.6 mg/mL collagenase D (Roche Products, Indianapolis, IN, USA), 50 μg/mL dispase I (Roche Products), 15 μg/mL hyaluronidase (Sigma, St Louis, MO, USA), 0.5 mg/mL bovine serum albumin (MP Biomedicals, Irvine, CA, USA) and 50 U DNase I (Sigma) for 1 h at 37°C, before centrifugation and plating. Initial plating in MEM-Dvaline, 10% fetal bovine serum (FBS) and 100 U/mL penicillin/streptomycin (P/S) onto a poly-L-lysine substrate without additional mitogens was followed 4–5 days later by enrichment performed using anti-Thy1.1-mediated complement lysis to remove the majority of contaminating fibroblasts. Cells were replated in DMEM/F-12, 10% FBS and 100 U/mL P/S and allowed to grow for an additional 4–6 days, when they were again subjected to Thy1.1-mediated complement lysis and grown in the same media for an additional 24–48 h prior to transplantation. Concurrently, a subset of cells were plated on to plastic coverslips and fixed for antigenic assessment at the same time as harvesting for transplantation. They were assessed as at least 75% double immunopositive for p75 and glial fibrillary acidic protein (GFAP), hence the fibroblast portion (assessed as fibronectin+/p75-negative) did not exceed 25%. Under these culture conditions (density of plating media, composition of plating media and plating substrate) we have previously established OECs readily expand, whereas Schwann cells expand very poorly (Richter & Roskams, 2008). Although the dissected nasal mucosa may contain a small percentage of Schwann cells derived from cranial nerves, when assessed under identical conditions *in vitro*, Schwann cells are also significantly smaller than OECs and demonstrate very different morphologies, mitosis and migration capabilities. Thus, any significant representation of Schwann cell contamination would be apparent upon visual inspection. Although we can not rule out a few contaminating Schwann cells from sensory nerves in the mucosa, this contribution is minimal.

LP-OECs were plated at a density of 5600 cells/cm^2 into T75 flasks for transplantation. Before transplantation, the cells from one T75 flask of LP-OECs were detached using 0.25% trypsin/1% EDTA, followed by washing in phosphate-buffered saline (PBS) and resuspension at a concentration of 100 000–120 000 cells/μL in DMEM/F-12. The total time from dissection to transplantation ranged from 11 to 14 days *in vitro*. Because initial experiments by using fresh prepared OECs indicated maintenance of phenotype after cryopreservation of cells (Ramer *et al.*, 2004a; and this study), some experiments used OECs that were cryopreserved.

Spinal cord injury and treatment

Animal procedures were performed in accordance with the guidelines of the Canadian Council for Animal Care and were approved by the Animal Care Committee of the University of British Columbia. A total of 28 out of 32 animals were used for behavioral and histological analysis.

Dorsolateral funiculus crush

Thirty-two adult male Sprague–Dawley rats (300–400 g) were immunosuppressed with cyclosporine A (10 mg/kg per day, i.p.; Novartis Pharmaceuticals, Mississauga, ON, Canada) 2 days before surgery and each day for the duration of the experiment. Rats were anesthetized with a mixture of ketamine hydrochloride (70 mg/kg, i.p.; Bimeda-MTC, Cambridge, ON, Canada) and xylazine hydrochloride (10 mg/kg i.p.; Bayer, Etobicoke, ON, Canada), a laminectomy was performed on the left side, exposing the fourth and fifth cervical segments. The dura was cut with microscissors to expose the spinal cord and the dorsolateral funiculus including the rubrospinal tract was crushed for 20 s with custom-designed fine surgical forceps at a depth of 2 mm, but otherwise as described previously (Ramer *et al.*, 2004a; Richter *et al.*, 2005). The distal blades of # 5 Dumont forceps were grounded to a width of 0.2 mm for the length of exactly 2 mm, allowing a reproducible insertion of a fine tip to the desired depth of 2 mm (see also Plunet *et al.*, 2008).

Cell transplantation

LP-OECs were transplanted into the spinal cord as described previously (Ramer *et al.*, 2004a; Richter *et al.*, 2005). LP-OEC slurries in DMEM/F-12 were drawn into the pulled glass pipette with a diameter of 60–80 μm attached to a Hamilton syringe. OECs were stereotaxically microinjected 1 mm rostral and caudal to the lesion site, dividing the suspension equally between these two points. A total of 1.5 μL of cell slurry was injected, so that each rat received a total of 150 000–180 000 cells. Control animals received the same volume of DMEM/F-12 injected at the same sites and at the same rate. The glass pipette remained in place for 5 min after each injection to ensure that cells remained in the spinal cord and were not withdrawn with the syringe. After injection, the pipette was slowly pulled back, the muscle repositioned and the skin closed with wound clips.

Cell body treatment

BDNF or vehicle was infused for 2 weeks via a cannula inserted chronically in the vicinity of the red nucleus, as described previously (Kobayashi *et al.*, 1997; Kwon *et al.*, 2002). In brief, after cell transplantation (while the rats were still anesthetized as above), a 28-gage cannula connected by silastic tubing to an osmotic minipump (Alzet no. 2002, 0.5 μL/h; Alzet, Palo Alto, CA, USA) was inserted stereotaxically 6.3 mm posterior to Bregma, 1.7 mm lateral (right) of midline and 6.5 mm deep from the dura. Animals treated with BDNF received 0.5 μg/μL/h of BDNF (gift from Regeneron Pharmaceuticals, Tarrytown, NY, USA) in a vehicle solution of 20 mM sterile PBS, 100 U of P/S and 0.5% rat serum albumin (Sigma-Aldrich; no. A-6272). Animals treated with vehicle received the vehicle solution alone. After insertion of the cannula and insertion of the osmotic minipump under the skin of the neck, the skin was closed with wound clips: see Fig. 1 and Table 1 for the experimental design and more details.

Behavioral testing

Cylinder test

Forelimb usage was videotaped in a clear Plexiglas cylinder (20 cm in diameter and 30 cm high) for 5 min (Liu *et al.*, 1999). The cylinder encourages rats to use their forelimbs for vertical exploration. A mirror was placed behind the cylinder to enable scoring of movements from

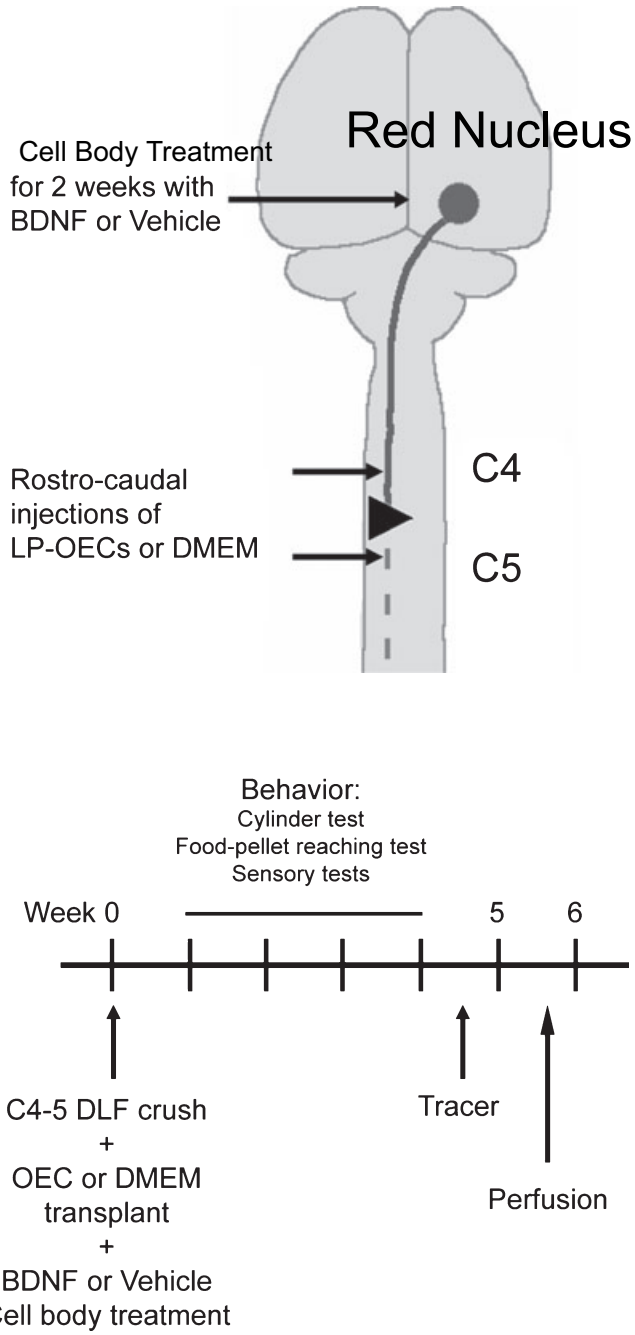

FIG. 1. Experimental design. The dorsolateral funiculus including the rubrospinal tract was axotomized at C4–C5. Olfactory ensheathing cells (OECs) derived from the lamina-propria (LP) of neo-natal mouse expressing GFP were injected rostrally and caudally (1 mm) to the lesion epicenter. DMEM was injected as control into the same locations. In combination with this spinal bridge, rubrospinal neurons were treated with brain-derived neurotrophic factor (BDNF) or vehicle (as control) for 2 weeks via a cannula inserted into the vicinity of the red nucleus.

all viewpoints. The behavior was scored frame by frame at a later time-point by a blinded rater. Forelimb use was scored as independent use of the left injured forelimb, independent use of the right forelimb and concomitant use of both forelimbs for contacting the wall of the cylinder during a full rear. To reflect a more accurate use of the left forelimb, the score was expressed as a percentage use of the 'left and both' forelimbs relative to the total number of left, right and both forelimb use.

TABLE 1. Number of animals used for histological and behavioral quantifications

Cell body treatment Spinal transplant	Vehicle DMEM	Vehicle LP-OECs	BDNF DMEM	BDNF LP-OECs
Histology	7	5	3	8
Cylinder test	8	5	4	7
Reaching task	6	4	4	6
Sensory tests	4	8	4	8

BDNF, brain-derived neurotrophic factor; LP, lamina-propia; OEC, olfactory ensheathing cell.

Food-pellet reaching task

The food-pellet reaching task used is adapted from Whishaw *et al.* (1993) and was simplified elsewhere (Chan *et al.*, 2005). In brief, the rats were trained before injury to reach through a 1-cm opening a food pellet placed in a dimple 2 cm away. The behavior was scored on a 10-point scale, reflecting the execution of sequential aspects for reaching (score 3), grasping (score 5) and retrieving (score 7) a food pellet (for details, see Chan *et al.*, 2005).

Sensory tests

The sensory testing has been detailed elsewhere (Ramer *et al.*, 2004b). The thermal threshold of forepaws was examined by using the plantar tester (Ugo Basile, Italy). Rats were placed in a designed cage in a glass floor over a moveable infrared generator. The infrared source was positioned under the center of the palmar forepaw. The time from stimulus onset to withdrawal was recorded for both forepaws. Mechanical threshold of forepaws was also examined using the plantar aesthesiometer (Ugo Basile, Italy). Rats were placed in a raised cage with a wire mesh floor over the stimulator unit. The vertical metal filament was applied to the center of the palmar surface of the forepaw, and upward force was increased from 1 to 50 g over 7 s. Force and latency at withdrawal were recorded for both forepaws. For both sensory tests, rats were tested weekly two–three times for each paw for 2–3 weeks before injury to ensure a control baseline, and for 4 weeks after injury and treatment.

Baseline behavior was measured before surgery, and all animals were tested weekly for 4 weeks after injury and treatment. To force rats to perform the food-pellet reaching task and to prevent any influences on other behavioral tests, all rats were fasted once a week the night preceding the testing day.

Labeling of rubrospinal axons and immunocytochemistry

Anterograde labeling of rubrospinal axons

Five weeks after spinal cord injury and treatment, rats were anesthetized with a mixture of ketamine hydrochloride (70 mg/kg, i.p.; Bimeda-MTC, Cambridge, ON, Canada) and xylazine hydrochloride (10 mg/kg i.p.; Bayer, Etobicoke, ON, Canada), and placed in a Kopf stereotaxic frame. Biotinylated dextran amine (BDA; 10 000 kDa molecular mass, 10% in sterile water, Molecular Probes, Eugene, OR, USA) was stereotaxically injected into the red nucleus at a rate of 30 nL/min by using a pulled glass pipette (diameter of 20 μm) glued to a Hamilton syringe. Coordinates were 6.1 mm posterior to Bregma, 0.7 mm lateral (right) of midline and 7 mm deep from the dura. The pipette remained in place for 10 min after injection to ensure that BDA was not withdrawn with the syringe.

Immunocytochemistry

Six weeks after injury and treatment, rats were anesthetized with a lethal dose of chloral hydrate (100 mg/kg, i.p.; BDH Chemicals, Toronto, ON, Canada) and perfused transcardially with PBS followed by phosphate-buffered, 4% paraformaldehyde (pH 7.4). The midbrain, cerebellum and cervical spinal cords were dissected, postfixed in 4% paraformaldehyde overnight, cryoprotected in 24% sucrose in 0.1 M phosphate buffer over 2–3 days, and frozen in isopentane over dry ice. Spinal cords were cut into 20-μm sections on a cryostat and stored at −80°C. Cervical segments C2 and C7 were cut into 20-μm sections in the coronal plane. Cervical segments from C3 to C6 were cut into 20-μm longitudinal sections in the horizontal plane. After cutting, frozen sections were thawed on a slide warmer for 30 min, rehydrated in 10 mM PBS three times for 5 min, and incubated with 10% normal donkey serum (in 0.1% Triton X-100) for 30 min to prevent non-specific binding. The following primary antibodies were used: chicken anti-GFP (anti-GFP, 1 : 1000; Chemicon), rabbit anti-GFP (anti-GFP, 1 : 1000; Chemicon), mouse anti-GFAP (anti-GFAP, 1 : 400; Sigma), mouse anti-neurofilament 200 (anti-NF, 1 : 400; Sigma), mouse anti-beta-III-tubulin (anti-tub, 1 : 400; Sigma), rabbit anti-serotonin transporter (anti-SERT, 1 : 500; ImmunoStar), sheep anti-tyrosine hydroxylase (anti-TH, 1 : 200; Chemicon), rabbit anti-calcitonin gene-related peptide (anti-CGRP, 1 : 500; Sigma), chicken anti-P0 (anti-P0, 1 : 100; AvesLabs) and chicken anti-myelin basic protein (anti-MBP, 1 : 100; AvesLabs). All primary antibodies were applied for 24 h at room temperature. Secondary antibodies (1 : 200, Jackson) raised in donkey, conjugated to Alexa 350, Alexa 488 and Cy3, and were applied for 2–3 h at room temperature. BDA was visualized by using Cy3- or AMCA-conjugated streptavidin (1 : 400, Jackson) applied for 2–3 h at room temperature. Sections were coverslipped in glycerol mounting liquid (Sigma).

Image analysis and quantification

Images were digitally captured with an Axioplan 2 microscope (Zeiss, Jena, Germany), a digital camera (QImaging, Burnaby, BC, Canada) and NORTHERN ECLIPSE software (Empix Imaging, Mississauga, ON, Canada). Digital images were then processed by using PHOTOSHOP 7.0 (Adobe Systems, San Jose, CA, USA), SIGMASCAN PRO (SPSS, Chicago, IL, USA) and MATLAB 6.5 (The MathWorks, Natick, MA, USA) softwares. The number of rubrospinal axons was quantified for each animal by measuring the number of BDA-traced axons in three cross-sections at C2. Rubrospinal regeneration was assessed by searching for the presence of traced axons throughout the spinal cord every other 20 μm in longitudinal (horizontal) sections. As we did not find any evidence for axonal regeneration, we eventually measured for each animal the distance between the terminal ends of the most caudal five rubrospinal axon tips and the lesion epicenter using the three best traced longitudinal sections of the rubrospinal tract in the dorsolateral funiculus. This represents a measure of rubrospinal axon retraction and possibly sprouting proximal to the injury. The efficacy of the axonal tracing was confirmed by counting the number of rubrospinal axons measured on three different cervical coronal sections at C2. Lesion area and cavity size were quantified for each animal by outlining the lesion area defined by GFAP immunoreactivity at three defined levels equally distributed between the dorsal root entry zone and the central canal, and calculating the total pixels in this area. GFAP immunoreactivity was evaluated by measuring the intensity within a 50-μm width area circumscribing the lesion site on these former three longitudinal sections and normalized on the analogous area on the contralateral uninjured side of the spinal cord. Immuno-positive cellular or axonal density measurements were adapted from Ramer *et al.* (2004b). In brief, images were captured from three sections (per animal) in the horizontal plane equally distributed between the dorsal root entry zone and the central canal. A Laplace transformation was applied, which enhances the edges of the immunoreactive axonal and cellular profiles. This step compensates for fluctuations in the brightness of the immunofluorescent signal. Subsequently a threshold (using the same throughout the experiment) was applied and an overlay generated, which selected preferentially the axonal profiles. The pixel area occupied by the thresholded axonal profiles was divided by the area (pixel number) of the selected measurement window to generate axonal density values (ratios). The same procedure was applied to the GFP-positive transplanted cells to obtain a measure of their density (strictly speaking the density of their cytoplasmic and nuclear borders) within the measurement window. Averaged axonal and cell density measurements from each treatment group were processed and plotted as a function of the distance from the lesion epicenter.

Statistical analysis

A two-way repeated ANOVA was used to detect interactions between treatment and distance for the axonal and cellular density, and to detect interactions between treatment and time course for the behavioral outcomes. A one-way ANOVA was used to detect differences in the axonal or cellular density at each given distance and to detect differences in behavioral outcome measurements at each time-point. A one-way ANOVA was used to detect differences between averaged density measurements as well as rubrospinal axon retraction and lesion area measurements according to the treatment. The data were further analysed using the *post hoc* Tukey–Kramer test. In the cases where the variables did not fit a normal distribution, the non-parametric Kruskal–Wallis ranked sum test was used, followed by a chi-square test. In all cases, significance was taken as $P < 0.05$.

Results

We reported previously some rubrospinal axon growth into LP-OEC grafts implanted into the lesion site, but no regeneration beyond (Ramer *et al.*, 2004a). Here, we choose to transplant highly enriched LP-OECs rostrally and caudally because we found recently that this reduces cavity formation, GFAP expression at the lesion boundary and enhances the growth of NF-positive axons into the lesion site (Richter *et al.*, 2005). In the present study we combined this improved transplantation approach with a 2-week infusion of BDNF into the vicinity of the cell bodies of the rubrospinal neurons in order to promote their regenerative propensity as well as functional recovery (see Fig. 1 and Table 1 for experimental design).

OECs fill the lesion, and reduce cavity and glial scar formation

Injury of the rat dorsolateral funiculus typically results in the formation of a lesion with cavitation that is surrounded by a margin of hypertrophic, GFAP-positive astrocytes. We defined this GFAP-negative area in the epicenter as 'lesion area', which in the experimental groups became filled with transplanted cells as well as cells invading from the roots and meninges. All three treatment groups (Vehicle–OEC, BDNF–DMEM, BDNF–OEC) appeared to have smaller lesion areas than our control group (Vehicle–DMEM; Fig. 2A), yet these measurements failed to reach significance ($P = 0.14$ BDNF–OEC group; Fig 3A). Transplantation of OECs

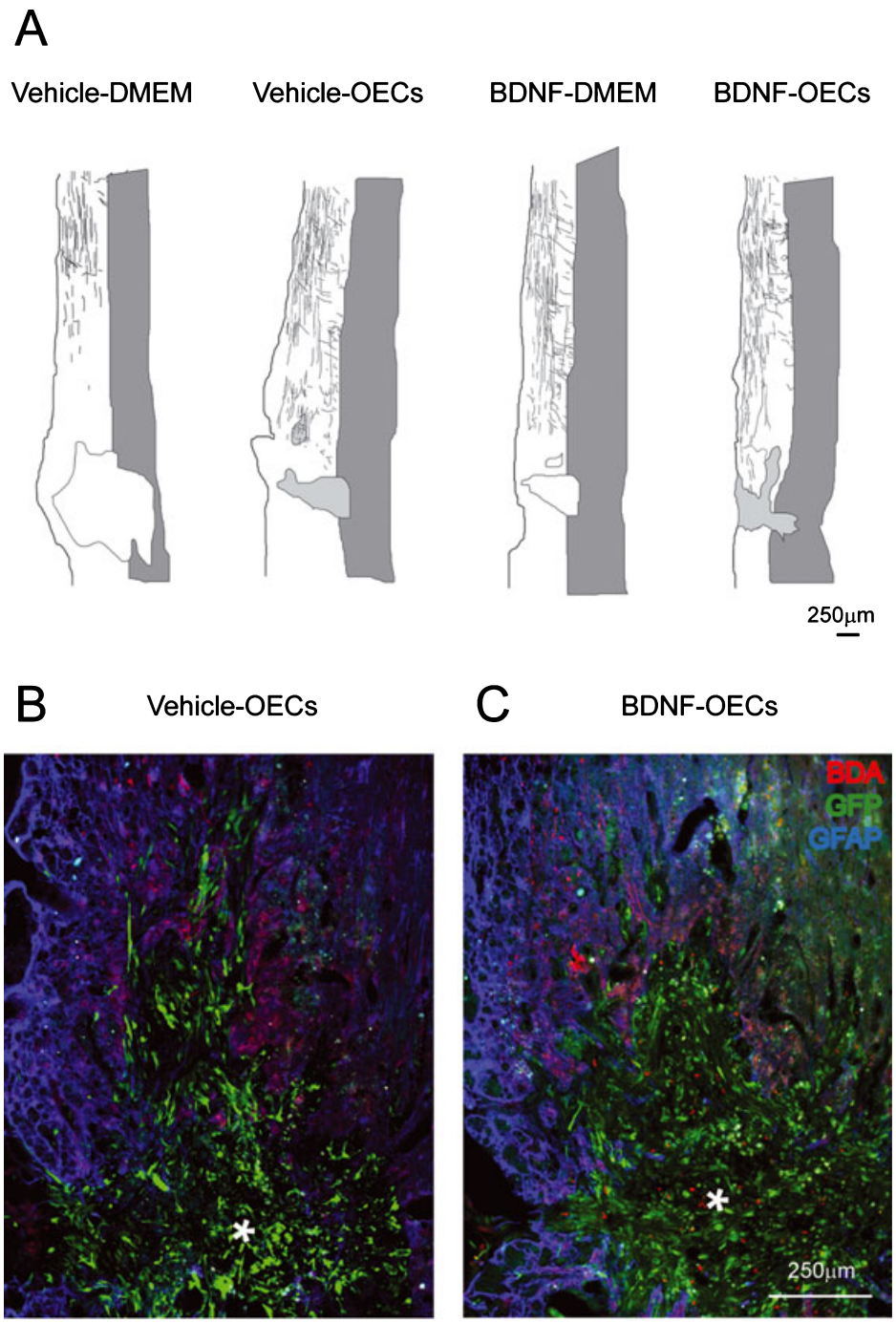

FIG. 2. Olfactory ensheathing cells (OECs) bridge the injured spinal cord, but rubrospinal axons do not regenerate. (A) Camera lucida of rubrospinal axons. Representative longitudinal spinal cord sections at the dorsolateral funiculus level for different treatments and their combinations. Note rubrospinal axons run along the white matter and grow into the rostral OECs graft (light gray area), but stop at the edge of the OEC transplants bridging the lesion site. Rubrospinal branching within the gray matter (dark gray area) is not represented. (B and C) Horizontal sections of the cervical spinal cord injury site immunostained for astrocytes [glial fibrillary acidic protein (GFAP) in blue], LP-OECs [green fluorescent protein (GFP) in green] and rubrospinal axons [biotinylated dextran amine (BDA) in red]. (B) Vehicle–OEC-treated rats, which received vehicle at the rubrospinal neuron level and OECs at the spinal cord level. (C) Brain-derived neurotrophic factor (BDNF)–OEC-treated rats. Neither treatment alone or in combination promoted regeneration of rubrospinal axons. The white asterisks in B and C indicate the lesion epicenters.

alone (Vehicle–OEC) or in combination with BDNF treatment of the rubrospinal cell bodies (BDNF–OEC) diminished the size of the cavity area (Figs 2 and 3B), which was also reflected by a decreased percentage of lesion area occupied by cavitation (Fig. 3C). Both groups receiving OECs (Vehicle–OEC and BDNF–OEC; Fig. 2B and C) showed reduced immunoreactivity for GFAP, indicative of less hypertrophy of reactive astrocytes (Fig. 3D) compared with the control group. Cell body treatment with BDNF combined with DMEM at the spinal cord resulted in a trend towards smaller cavity areas and GFAP immunoreactivity; however, these parameters failed to reach significance when compared with the control group (Fig. 3B–D). Moreover, OEC survival was not affected by cell body treatment with BDNF

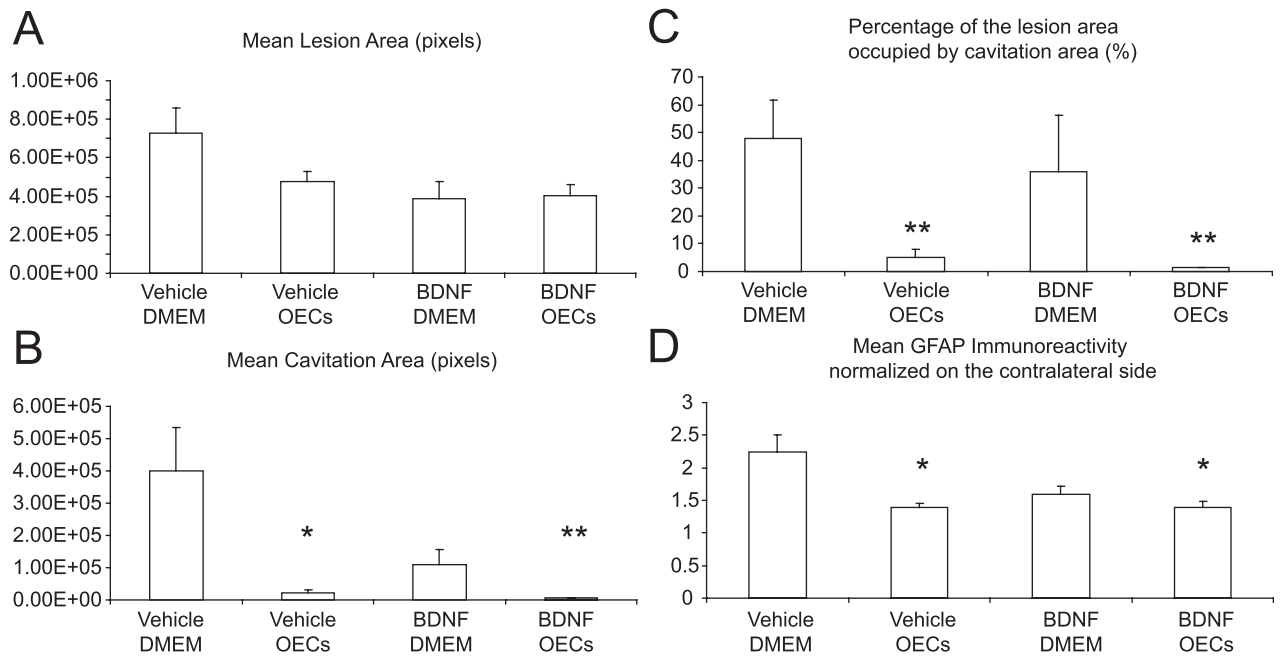

FIG. 3. LP-olfactory ensheathing cells (OECs) irrespective of the cell body treatment decreased lesion area, cavity formation and glial scar formation. (A) Mean lesion area (GFAP-negative area). (B) Mean cavitation area (background area within the lesion site). (C) Percentage of lesion site occupied by cavity formation. (D) GFAP immunodensity along the lesion site normalized on the contralateral non-injured side. Error bars indicate SEM [Kruskal–Wallis, $P < 0.01$: *compared with Veh–DMEM; **compared with brain-derived neurotrophic factor (BDNF)–DMEM and Veh–DMEM].

(Fig. 4, two-way repeated ANOVA, $P > 0.95$ for the treatment × distance interaction). In summary, the minimal neuroprotection (inferred by lesion area measurement) elicited by the combination of OECs with BDNF was not different from OEC or BDNF treatment alone, i.e. the effects were not additive. Similarly, the combination of OECs and BDNF did not have an additive effect on astrocytic hypertrophy, cavity formation and percentage of the lesion area occupied by cavitation. Thus, transplantation of OECs alone appears responsible for the observed beneficial effects on GFAP immunoreactivity and cavity formation.

Prevention of rubrospinal axon retraction

We next examined the regeneration of rubrospinal axons by labeling them with the anterogradely transported tracer BDA. Typically rubrospinal axons retract after axotomy (Ye & Houle, 1997; Jin *et al.*, 2002) and, in our control condition (Veh–DMEM; Fig. 2A) involving rostral/caudal DMEM injections, most rubrospinal axons ended about 1700 μm (± 323 μm) rostral to the lesion center. In contrast, in all three experimental conditions (BDNF–DMEM, Veh–OECs and their combination BDNF–OEC), the bulk of these rubrospinal axons extended to about 500 μm rostrally of the epicenter ($P < 0.01$; Figs 2A and 5A). While we can not always distinguish atrophic retracting axons from newly sprouting axons, it is important to note that most anterogradely traced axons ended within the host tissue and were only rarely seen in the proximity of transplanted cells. The straight course of many of these axons favors the interpretation that these had retracted. Important to note, these differences in rubrospinal axon profiles at the rostral lesion edge were not due to differences in the efficacy of anterograde tracing. The number of rubrospinal axons filled with BDA counted on coronal (i.e. cross-) sections taken at C2 was lower in the control group, but this did not reach significance ($P = 0.11$; Fig. 5B) between the three treatment groups and the Vehicle–DMEM-treated control group. Hence, all three treatments (i.e. BDNF–DMEM, Veh–OEC or their combination) appeared to prevent the retraction of rubrospinal axons from the lesion epicenter ($P < 0.01$; Fig. 5A), but neither treatment condition promoted regeneration of rubrospinal axons through and beyond the transplanted LP-OECs.

Despite these limited effects on rubrospinal regeneration, OECs promoted sprouting of various axonal populations (NF-200 and/or tub-positive structures) into the OEC-filled lesion site irrespective of the cell body treatment (Fig. 6; one-way ANOVA, $P = 0.43$). We therefore examined the sprouting of identified supra-spinal axons and peripheral afferents. The immunoreactivity for supra-spinal fibers, such as serotonergic (SERT-positive fibers; Fig. 7A–C) and noradrenergic (TH-positive fibers; Fig. 7D–F) fibers was higher at the rostral lesion transplant interface than caudal to the transplant in all groups; however, only a few axons grew into the engrafted OECs. Axonal density measurements revealed significantly more SERT- and TH-positive fibers rostral to the lesion in OEC treated spinal cords (Veh–OEC and BDNF–OEC) compared with controls. The density of peripheral afferents positive for substance-P (Sub-P) was higher caudal to the lesion site, but this phenomenon was not significantly different among the groups (Fig. 7G–I). In contrast, small-diameter peptidergic nociceptive fibers containing CGRP were massively increased in the OEC transplanted rats, both at the cranial and caudal lesion margins as well as inside the epicenters of the transplants (Fig. 7J–L). While the density of CGRP-positive fibers was highest inside the transplant with Vehicle–OEC treatment, the combination of OECs with BDNF resulted in significantly more CGRP-positive axons extending from 1.5 mm rostral to the lesion into the OECs grafts (Fig. 7K and L). Thus, OEC grafts had pronounced effects on the sprouting of SERT-, TH- and CGRP-positive axons, and the growth of the latter population was enhanced

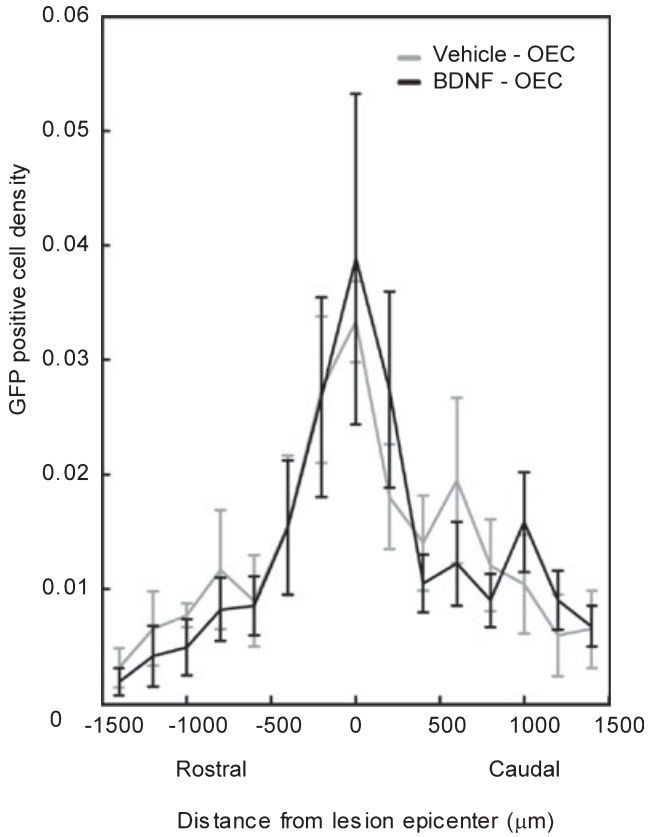

FIG. 4. LP-olfactory ensheathing cells (OECs) survival is not affected by the cell body treatment. OEC density as a function of the distance from the lesion epicenter from brain-derived neurotrophic factor (BDNF)–OECs- and Veh–OECs-treated rats. Error bars indicate SEM (two-way repeated-measures ANOVA revealed no treatment × distance interaction, $P = 0.985$, or a treatment effect, $P = 0.959$). GFP, green fluorescent protein.

by the application of BDNF to the vicinity of the red nucleus in the midbrain.

Functional outcomes

To assess the behavioral outcomes of our treatments on forelimb function on the left (injured) side of the spinal cord, we evaluated the usage of the left forelimb for the cylinder test and the food-pellet reaching task. Figure 8A illustrates the time course of forelimb usage during vertical exploration of a Plexiglas cylinder. Conventionally the usage of the left forelimb is scored as 'left plus both' together. Typically, rats rear in about 25% with the left or right forelimb, and in 50% with both simultaneously. Hence, control values were found about 75–80% for all groups before spinal cord injury as expected from the literature (Liu *et al.*, 1999; Schallert *et al.*, 2000). After spinal cord injury, the forelimb usage dropped significantly to 30–40% in rats, who received control treatment or any of the two mono-treatments. Surprisingly, it dropped drastically to 5% in rats treated with the combinatorial treatment of BDNF and OECs, indicating that the left forelimb was hardly used at all and 95% of the rears were performed with the right paw. This diminution in the use of the left forelimb in BDNF–OEC-treated rats was observed for the first 2 weeks after injury (equivalent to the flow period of the osmotic minipump) and subsequently the usage gradually returned to the levels of other groups.

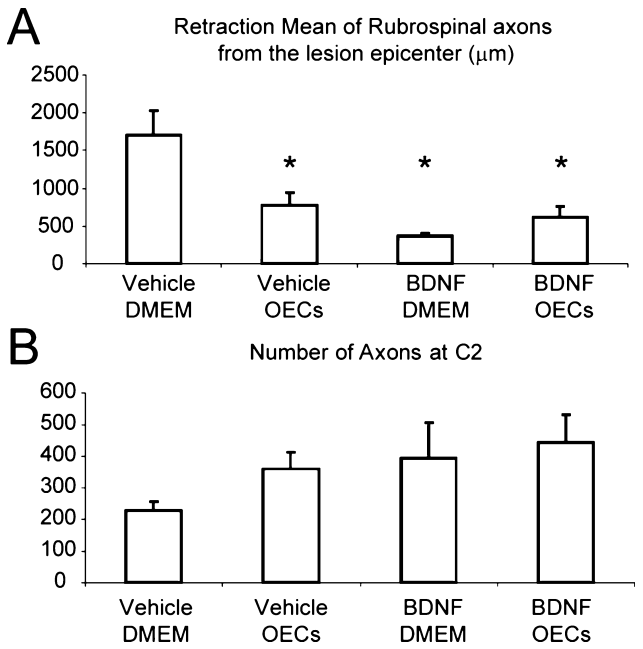

FIG. 5. All three treatments, olfactory ensheathing cell (OEC) transplantation, cell body treatment with brain-derived neurotrophic factor (BDNF) as well as their combination prevented retraction of rubrospinal axons from lesion epicenter. (A) Distance of rubrospinal axonal terminals from the lesion epicenter as a function of different treatments. (B) Number of rubrospinal axons at the cervical segment C2 rostral to the lesion. Error bars indicate SEM (*one-way ANOVA, $P < 0.05$; compared with the control group Veh–DMEM). Note that although the combinatorial therapy BDNF–OECs did not promote regeneration, it increased the number of axons at C2 and prevented the retraction of rubrospinal terminals from the lesion epicenter.

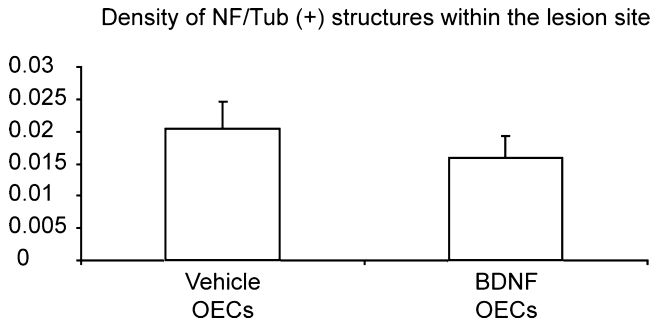

FIG. 6. Olfactory ensheathing cells (OECs) promoted regeneration of unidentified thin and thick axons irrespective of the cell body treatment. Axonal density of thin and thick axons [neurofilament (NF)-200- and beta-III-tubulin (tub)-positive structures, respectively] throughout the OECs graft according to the cell body treatment. Note that although BDNF–OEC treatment decreased the axonal density slightly, no significant differences were reported between both groups (ANOVA, $P = 0.437$). Error bars indicate SEM.

The rats were also tested for a more sophisticated motor task involving proximal as well as distal muscles: the food-pellet reaching task (Chan *et al.*, 2005). Figure 8B illustrates the time course of motor skill changes for the food-pellet reaching task scaled on 10 points. Before injury, all rats were able to reach forward, touch, grasp and retrieve the food pellet (average score of 8, see Chan *et al.*, 2005 for details of the scale). After spinal cord injury, control rats as well as OEC-treated rats were able to reach forward and touch the pellet, but failed to grasp it (score 3) during the first 3 weeks post-injury. By 4 weeks they

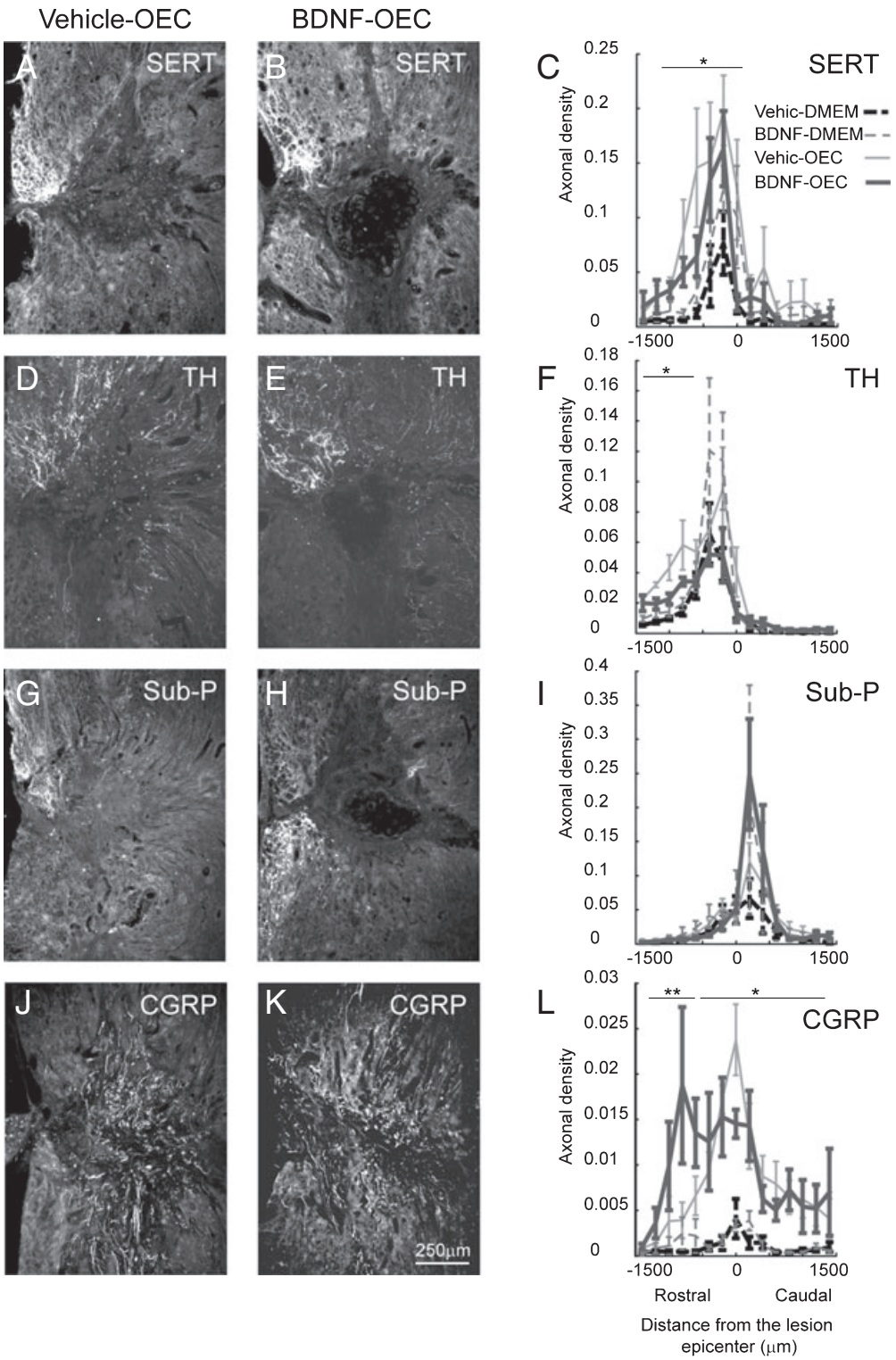

FIG. 7. Absence of regenerative sprouting of supraspinal axons and peripheral afferents into engrafted LP-olfactory ensheathing cells (OECs), except small diameter peptidergic fibers. OECs alone (left column) or in combination with brain-derived neurotrophic factor (BDNF; middle column) increased immunoreactivity of marker of serotoninergic re-uptake vesicles (SERT-; A–C) and tyrosine hydroxylase (TH-; D–F; marker of noradrenergic fibers) positive fibers rostral to the lesion site, but promoted poor or no regeneration of these fibers into the OECs graft. Similarly, OECs increased immunoreactivity of substance-P (Sub-P)-positive fibers (G–I; marker of peripheral afferent terminals) caudal to the lesion site, but very little into the OECs graft. Only calcitonin gene-related peptide (CGRP)-positive fibers (J–L; marker of peptidergic small diameter peripheral afferent terminals) grew into the engrafted OECs. Right column: axonal density of SERT-, TH-, Sub-P- and CGRP-positive fibers as a function of the distance from the lesion epicenter according to the treatment. Note significant increases in CGRP axonal density in BDNF–OEC- and Veh–OEC- treated rats in comparison with BDNF–DMEM- (dotted light gray line) and Veh–DMEM- treated ones. Error bars indicate SEM. Two-way repeated-measures ANOVA ($P < 0.05$) revealed a time × distance interaction for SERT, TH and CGRP fibers. One-way ANOVA, $P < 0.05$: *(BDNF–OECs and Veh–OECs) compared with (Veh–DMEM and BDNF–DMEM); **BDNF–OECs compared with three other groups.

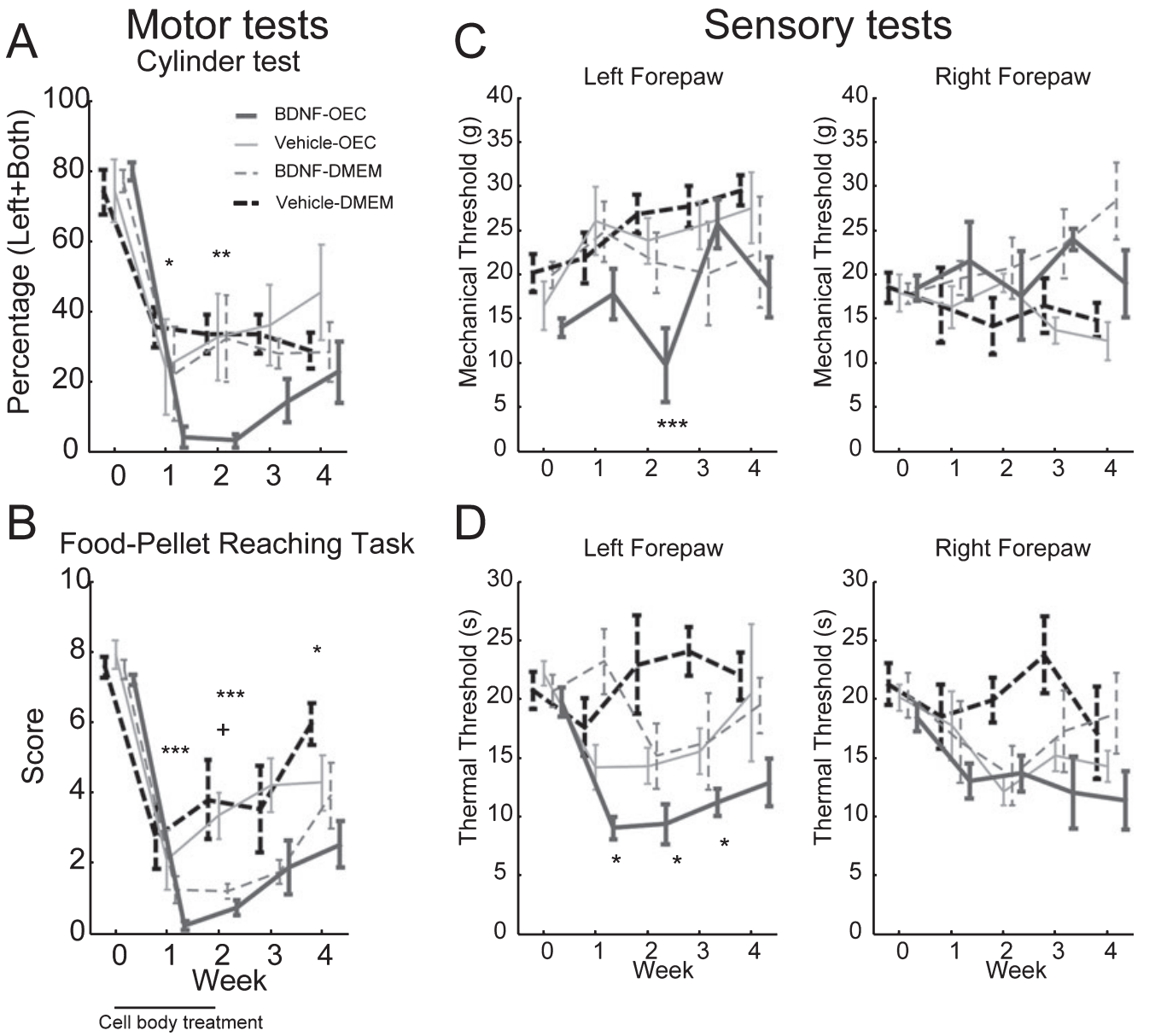

FIG. 8. Combinatorial therapy delayed and diminished functional recovery. (A) Time course of percentage changes of use of left and both forelimbs to contact the wall for the cylinder test before and after spinal cord injury and treatment. (B) Time course of changes in the motor performance for reaching, grasping and retrieving a food pellet. (C) Time course of changes in mechanical thresholds of left and right forepaws. (D) Time course of changes in thermal thresholds of left and right forepaws. For the left forepaw, two-way repeated-measures ANOVA ($P < 0.05$) revealed a time and treatment effect for the cylinder test, the food-pellet reaching task and for the left forelimb for both sensory tests; one-way repeated ANOVA, $P < 0.05$: *brain-derived neurotrophic factor (BDNF)–olfactory ensheathing cells (OECs) compared with control group Veh–DMEM; **BDNF–OECs compared with all groups; ***BDNF–OECs compared with (Veh–DMEM and Veh–OECs), and $^{+}$BDNF–DMEM compared with (Veh–DMEM and Veh–OECs).

improved and succeeded to grasp (score 5) and sometimes even retrieve (score 7) the food pellet. In contrast, BDNF–OEC-treated rats were significantly more impaired after injury compared with the control group and often merely able to lift their left injured forelimb but not to reach forward (average score of 2) during the 4 weeks of observation. While BDNF-treated rats were also significantly more impaired during the second and third week in comparison to OEC-treated and control rats, they recovered and were no longer significantly different from the OEC-treated ($P > 0.95$) and control ($P = 0.3$) rats by 4 weeks. These observations indicate that the functional impairments observed in the cylinder and reaching tests were aggravated by the combination of OEC transplants and BDNF.

Because of this delay and diminution in the functional motor recovery of the left injured forelimb, we tested whether this might be due to a sensory alteration in the forepaws. Figure 8C and D illustrates time courses of sensory thresholds to mechanical and thermal stimulation before and after spinal cord injury and treatment. Although BDNF-treated or OEC-treated rats presented a decreased mechanical and thermal threshold in comparison to control rats ($P = 0.26$ and $P = 0.2$ for weeks 2 and 3, respectively, in Fig. 8D), only BDNF–OEC-treated rats showed a significant diminution in sensory thermal thresholds for the duration of the cell body treatment in comparison with the control rats, but this was no longer different from the other groups by 4 weeks after spinal cord injury and treatment ($P = 0.07$).

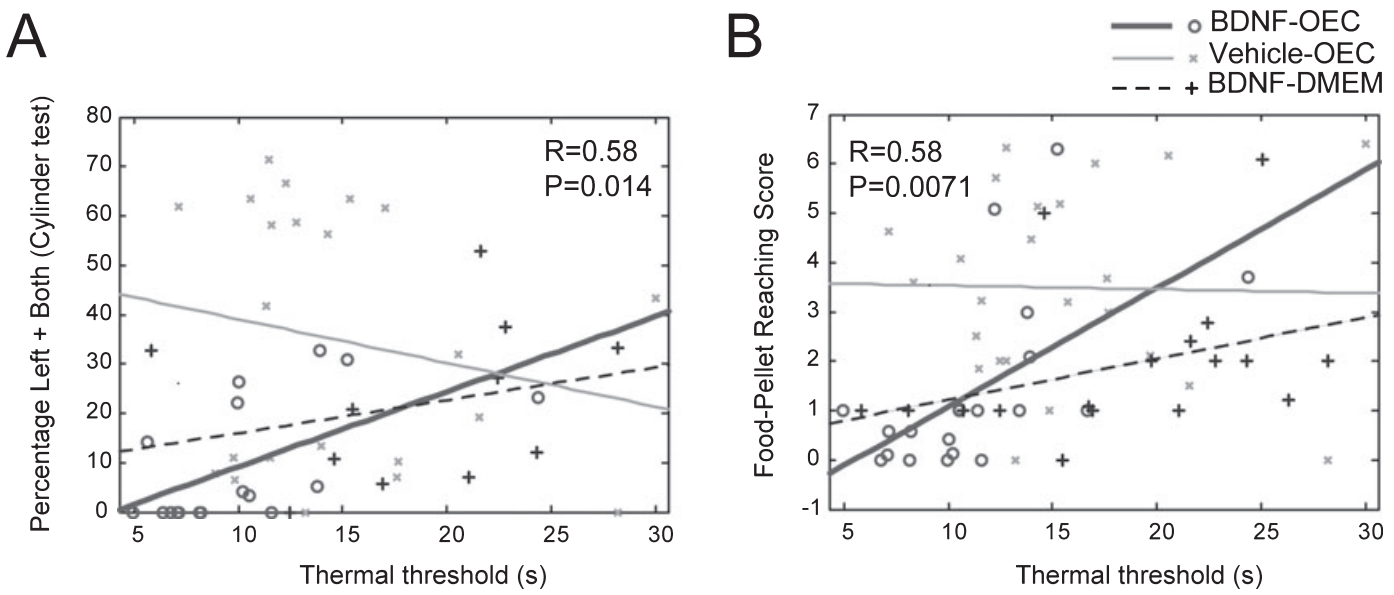

FIG. 9. Poor motor performances are strongly correlated to forepaw hypersensitivity in brain-derived neurotrophic factor (BDNF)–olfactory ensheathing cells (OECs)-treated rats. Correlation between motor performances for the cylinder test (A) or the food-pellet reaching task (B) and thermal thresholds in individual BDNF–OECs-, Veh–OECs- and BDNF–DMEM- treated rats. Significant correlation coefficients only for BDNF–OEC-treated rats (included in graphs).

Plotting the motor scores against the sensory thresholds revealed a strong correlation ($R = 0.58$, $P < 0.01$) between poor motor performances and low thermal thresholds in individual BDNF–OEC rats (Fig. 9). Thus, the delay and diminution in functional recovery observed in BDNF–OEC rats appeared to correlate with an alteration in mechanical and thermal sensations.

Discussion

Combinatorial treatments are increasingly favored to address the multifaceted problems occurring following spinal cord injury (Lu *et al.*, 2003; Nikulina *et al.*, 2004; Pearse *et al.*, 2004; Fouad *et al.*, 2005; Steinmetz *et al.*, 2005). Here we report for the first time the potential for detrimental effects of a combinatorial therapy. We combined transplantation of highly enriched LP-derived OECs (LP-OECs) into cervical spinal cord injury sites of rats with the infusion of BDNF to the red nucleus. All treatment groups using BDNF alone, OECs alone and BDNF–OECs tended to reduce the lesion size of the spinal cord, and the both groups with OECs reduced cavitation and astrocytic hypertrophy. Each treatment alone as well as in combination increased the number of rubrospinal axons rostral to the lesion site, however, no group showed rubrospinal axon regeneration through the OECs into the distal spinal cord. Somewhat surprisingly the combinatorial treatment, in addition to showing no further benefits beyond single treatment, impaired functional recovery of the forelimb on the injury side (left), which correlated with hypersensitivity of the forepaw.

The neuroprotective effects of transplanting neonatal LP-OECs alone were only minimal, which is similar to previous observations using adult rat OB-derived OECs in the injured dorsolateral funiculus in Fischer rats (Ruitenberg *et al.*, 2003). However, after transduction of OB-OECs with BDNF and/or NT-3-expressing viral vectors, they reduced lesion volumes and preserved the spinal cord cytoarchitecture in the injured dorsolateral funiculus (Ruitenberg *et al.*, 2003). Our combination of LP-OECs with BDNF infusion to the midbrain failed to reach significant neuroprotective effects despite the trend with each single treatment, indicating no apparent additive effects.

All three treatment groups showed more rubrospinal axons in close vicinity of the epicenter, which may be due to less axonal retraction/dieback and/or more sprouting than in the control group. It is not possible to distinguish these two scenarios in our experiments. The straight course of many rubrospinal axons in their original localization within the tract favors the interpretation of axonal protection (i.e. less retraction) rather than retraction and regrowth. Again, the effects of BDNF plus OECs were not additive, and the prevention of retraction was equally good with BDNF or OEC application alone. Rubrospinal axons were found intermingled between the transplanted cells at the site of OEC injection proximal to the injury, but not growing along the cells that filled the lesion site. While we cannot discern whether transplanted cells might have migrated between the axonal stumps of the rubrospinal tract, and/or some rubrospinal axons might have sprouted between the transplanted cells, there was no evidence for rubrospinal regeneration into or beyond the injury site. These findings are in line with the observations of Ruitenberg and co-workers, who found no sprouting of rubrospinal axons into adult OB-OEC-filled lesion sites, unless these were transduced to express trophic factors (Ruitenberg *et al.*, 2003). In our previous studies with LP-OECs (Ramer *et al.*, 2004a) we observed some rubrospinal axons among LP-OECs in the lesion site, which might be attributed to the different injection modes: proximal and distal to the injury in the present study as opposed to into the injury site in the previous study (Ramer *et al.*, 2004a) or attributed to the different growth factors made by early passage OECs compared with later passage OECs (Pastrana *et al.*, 2006; Au *et al.*, 2007; Franssen *et al.*, 2007). Our BDNF treatments here failed to stimulate rubrospinal axon growth into the transplants. There are most likely differences between the neurotrophic/tropic effects of BDNF overexpressed by transplanted cells (Ruitenberg *et al.*, 2003) vs. BDNF that is applied to the cell bodies and possibly released by the growing rubrospinal axons. BDNF infusion to the red nucleus increased BDNF mRNA in these neurons (Kobayashi *et al.*, 1997). In the first scenario, the

transplanted cells may create a gradient and BDNF may act as a neurotropic attractant in the lesion center, while in the latter scenario the source would be the axons of the proximal tract themselves, the gradient reversed and such attraction into the lesion unlikely. Nevertheless, in our previous studies we observed stimulation of rubrospinal axon growth into peripheral nerve transplants with BDNF cell body treatment (Kobayashi *et al.*, 1997; Kwon *et al.*, 2002), while in contrast no stimulation of rubrospinal sprouting into the OEC-filled lesion sites was apparent with this treatment in the present study. The survival time in these former studies was longer (> 10 weeks) then in the present study, which could have played some role. While longer survival times might have resulted in more regeneration, we feel that time for regeneration is not the main reason for these differences, as in our hands some rubrospinal axons regenerate into peripheral nerve transplants within a month (unpublished data, W.T.). These findings emphasize important differences between these two types of bridges, including the fact that our OECs were: (1) an enriched population of OECs rather than a mixture of cells with extensive extracellular matrix encountered in a nerve; (2) randomly oriented rather than aligned by long basal lamina tubes; (3) a mouse-to-rat xenograft that may stimulate an adverse immune response rather than a nerve autograft; and (4) a possibly weaker source of trophic/tropic factors than peripheral nerve grafts.

A recent report by Xiao *et al.* (2007) claimed extensive rubrospinal regeneration after transplantation of a cell line derived from human adult olfactory neuroepithelial progenitors cells cultured for up to 40 passages. While these cells likely arose from epigenetic events that produced an immortalized line (now commercialized by RhinoCytes), primary cultures of OECs do not show sphere-forming behavior and bear little similarity with these immortalized cells. We eagerly await an independent replication of the extensive rubrospinal regeneration claimed by these authors.

We failed to find functional recovery/compensation in a cylinder and reaching task by newborn mouse LP-OEC transplants in contrast to previous studies that reported behavioral improvement in a rope-walking task after adult rat OB-OECs transplants (Li *et al.*, 1997, 1998; Ruitenberg *et al.*, 2003; Sasaki *et al.*, 2004). This difference might be due to intrinsic differences between cell types (LP vs. OB; Au & Roskams, 2003; Richter *et al.*, 2005) or the age (neonatal vs. adult donors; Lipson *et al.*, 2003), or xenotransplant side effects and the behavioral test applied. Unexpectedly, the combination of OECs transplants with BDNF treatment of rubrospinal neurons impaired and delayed functional recovery. This apparent motor impairment correlated with sensory hypersensitivity for the duration of the cell body treatment and increased the density of CGRP-positive axons in the spinal cord proximal to the lesion in the combined BDNF–OEC treatment group. Although mechanical and thermal hypersensitivity has been reported after adult rat OB-OECs transplantations following a photochemical lesion of the spinal cord (Verdu *et al.*, 2003; Lopez-Vales *et al.*, 2004), we did not find altered sensitivity thresholds in our LP-OEC (vehicle–OECs) alone transplanted group. Neither did we observe lowered thresholds after BDNF infusion alone (BDNF–DMEM). Hence, the lowered sensory thresholds observed in our combined OEC–BDNF group were due to an unexpected combinatorial effect. We can only speculate on the possible mechanism. It is possible that the combined group sees higher levels of trophic factors at the site of spinal cord injury than the single treatment groups. For example, adult hamster and rat OB-OECs are known to express different types of neurotrophic factors, such as BDNF and nerve growth factor (NGF) (Boruch *et al.*, 2001; Lipson *et al.*, 2003; Sasaki *et al.*, 2006b; Wang *et al.*, 2006; Pastrana *et al.*, 2007), and this may be the case for LP-OECs. In addition, the exogenous delivery of BDNF at the red nucleus level increases the mRNA expression of BDNF by rubrospinal neurons (Kobayashi *et al.*, 1997), possibly increasing spinal BDNF delivery via axonal transport to their terminals. Although the literature is conflicting, BDNF has been implicated in sensitization of dorsal horn neurons (Kerr *et al.*, 1999; Mannion *et al.*, 1999; Heppenstall & Lewin, 2001; Pezet *et al.*, 2002; Coull *et al.*, 2005), and the role of NGF in pain mechanisms is well established (Christensen & Hulsebosch, 1997; Pezet *et al.*, 2001; Priestley *et al.*, 2002; Pezet & McMahon, 2006). Moreover, the infusion of BDNF might have reached the rostroventral medulla where it might have worsened nociceptive mechanisms, as recently shown with BDNF infusions into this area (Guo *et al.*, 2006). While BDNF infusion to the red nucleus may not have had an effect on sensory thresholds in the absence of cell transplantation, such a mechanism involving the medulla might have enhanced the sensitization effect of dorsal horn neurons by OECs transplants.

In conclusion, although our transplants of highly enriched LP-OECs bridged the injured spinal cord and reduced astrocyte hypertrophy, they failed to promote regeneration of rubrospinal axons through and beyond the lesion site. In addition, the combination of highly enriched OECs with BDNF infusion to the red nucleus diminished and delayed the functional recovery, which correlated with lowered sensory thresholds in the forepaw. These adverse effects of a combinatorial treatment were unexpected and contrast strongly with the beneficial effects reported by other combinations (Lu *et al.*, 2003; Nikulina *et al.*, 2004; Pearse *et al.*, 2004; Fouad *et al.*, 2005; Steinmetz *et al.*, 2005). BDNF infusion into the midbrain is a useful proof or principle to enhance axonal growth (Kobayashi *et al.*, 1997; Kwon *et al.*, 2002), but not likely of therapeutic potential in its present form as it may delay fine motor recovery. Alternatives for the enhancement of the intrinsic growth response of these neurons are needed that are not likely to elicit adverse effects especially in combinations. Thus, the observed changes in motor and sensory function after combination therapy highlight the necessity for extensive sensory and motor testing of candidate combination strategies that may have demonstrated some benefits alone.

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Abbreviations

BDA, biotinylated dextran amine; BDNF, brain-derived neurotrophic factor; CGRP, calcitonin gene-related peptide; FBS, fetal bovine serum; GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein; LP, lamina-propria; NF, neurofilament; NGF, nerve growth factor; OB, olfactory bulb; OEC, olfactory ensheathing cells; P/S, penicillin/streptomycin; PBS, phosphate-buffered saline; SERT, serotonin transporter; Sub-P, substance-P; TH, tyrosine hydroxylase; Tub, beta-III-tubulin; Veh, vehicle.

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