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Protection of Corticospinal Tract Neurons After Dorsal Spinal Cord Transection and Engraftment of Olfactory Ensheathing Cells

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Abstract

Transplantation of olfactory ensheathing cells (OECs) into the damaged rat spinal cord leads to directed elongative axonal regeneration and improved functional outcome. OECs are known to produce a number of neurotrophic molecules. To explore the possibility that OECs are neuroprotective for injured corticospinal tract (CST) neurons, we transplanted OECs into the dorsal transected spinal cord (T9) and examined primary motor cortex (M1) to assess apoptosis and neuronal loss at 1 and 4 weeks post-transplantation. The number of apoptotic cortical neurons was reduced at 1 week, and the extent of neuronal loss was reduced at 4 weeks. Biochemical analysis indicated an increase in BDNF levels in the spinal cord injury zone after OEC transplantation at 1 week. The transplanted OECs associated longitudinally with axons at 4 weeks. Thus, OEC transplantation into the injured spinal cord has distant neuroprotective effects on descending cortical projection neurons.

Keywords

olfactory ensheathing cells; spinal cord injury; apoptosis; corticospinal neurons; cell transplantation; neuroprotection

INTRODUCTION

Disruption of long axonal tracts in the spinal cord results in motor and sensory impairment below the level of the lesion. A number of studies (Li et al., 1997, 1998; Ramon-Cueto et al., 1998, 2000; Imaizumi et al., 2000; Polentes et al., 2004; Sasaki et al., 2004; Garcia-Alias et al., 2005; Ruitenberg et al., 2005) have demonstrated that structural and functional repair of the spinal cord occurs after transplantation of olfactory ensheathing cells (OECs) obtained from the olfactory bulb. Clinical investigations for SCI using OEC engraftment are in progress (Senior, 2002; Huang et al., 2003; see Reier, 2004, for overview of cell-based therapies in SCI). Although there is considerable evidence suggesting that, under appropriate cell preparation and transplantation conditions, functional outcome in experimental SCI can be enhanced by OEC transplantation, questions still remain with regard to cellular mechanisms responsible for improvement in functional outcome. Suggested spinal mechanisms include long tract regeneration (Li et al., 1997; Ramon-Cueto et al., 2000), axonal sparing and neuroprotection (Plant et al., 2003), sprouting and plasticity associated with novel polysynaptic pathways

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(Keyvan-Fouladi et al., 2002; Bareyre et al., 2004), recruitment of endogenous Schwann cells (Takami et al., 2002; Boyd et al., 2004; Ramer et al., 2004), and remyelination (Franklin et al., 1996; Imaizumi et al., 2000; Sasaki et al., 2004).

A recent study demonstrates that primary motor cortex (M1) pyramidal neurons undergo apoptotic cell death after axotomizing SCI (Hains et al., 2003). Moreover, earlier work has shown that corticospinal neurons become atrophic after spinal cord transection (McBride et al., 1989). In the present work, we describe the supraspinal effects of OEC transplantation on apoptosis and cell survival of corticospinal tract (CST) neurons within the M1 cortex after transection of their axons in the spinal cord. Our results indicate that apoptosis of primary motor cortical neurons is reduced and that cortical neuronal density is increased after OEC transplantation. Enhanced levels of BDNF were observed in the OEC transplanted lesion. Thus, transplantation of OECs into injured spinal cord has a neuroprotective effect on corticospinal neurons. The relative contribution of this effect to the observed functional improvement after OEC transplantation is uncertain, but these data indicate that OEC transplantation results in a larger pool of surviving corticospinal neurons.

MATERIALS AND METHODS

Isolation and Characterization of Donor OECs

Freshly isolated donor OECs were obtained using a procedure that relies on differential dissociation and attachment rates of subtypes of cells to serially remove other cell types and obtain a highly pure population of OECs. Olfactory bulbs were removed from 4- to 8-week-old transgenic rats expressing GFP ("green rat" CZ-004, SD-Tg (Act-EGFP) CZ-004Os; SLC, Shizuoka, Japan). The outer nerve layer of the olfactory bulb was dissected free of meninges and white matter and dissociated enzymatically with collagenase A and D and papain. A detailed description of OEC preparation can be found in Sasaki et al. (2004). The cells were suspended in DMEM (3×10^4 cells/ μ l) before transplantation and sham injections were made with DMEM alone. Immediately after transplantations were completed, the remaining OECs were cultured for 3–7 days and immunostained for p75, S-100, and GFAP with Hoechst nuclear staining.

Dorsal Funiculus Transection

Experiments were carried out in accordance with National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals, and the Yale University Institutional Animal Care and Use Committee approved all animal protocols. A T9 dorsal transection was made in adult female Sprague-Dawley (SD) rats ($n = 46$) (150–179 g) with aseptic technique as previously described in detail (Sasaki et al., 2004).

OEC Transplantation

Immediately after transection of the dorsal funiculus, GFP-expressing rat OECs ($n = 23$) or DMEM ($n = 23$) were injected into the dorsal funiculus using a drawn glass micropipette (Sasaki et al., 2004). Two injections were made at approximately 0.5 mm rostral and two at 0.5 mm caudal to the lesion at depths of 0.7 and 0.4 mm and an additional cell injection was made within the transection site for a total of five injections (1.0 μ l per site; 3.0×10^4 cells/ μ l for a total of 1.5×10^5 cells transplanted per rat). Gelfoam (Pharmacia and Upjohn, Kalamazoo, MI) impregnated with 5 μ l of Fluorogold (FG; 4% w/v in saline, pH 7.4; Molecular Probes, Eugene, OR), was placed into the epicenter of the lesion cavity (Hains et al., 2003).

Immunohistochemistry Procedures

Animals (n = 20) were perfused transcardially with 0.9% saline, followed by 4% paraformaldehyde in 0.14 M Sorensen's buffer; brain and spinal cord tissue were cryo-protected overnight at 4°C in 30% sucrose. Cryostat sections (12 µm; sagittal) of the spinal cord were incubated with antibodies against neurofilament (NF; 1:1,000; Sigma, St. Louis, MO), anti-monoclonal Ankyrin-G (AnkG; 1:60; EMD Biosciences, San Diego, CA), and anti-polyclonal Caspr (1:300, Rasband et al., 1999, a gift from Dr. M. Rasband, University of Connecticut Health Center, Farmington, CT). Details of immunostaining and imaging procedures can be found in Sasaki et al. (2004).

Tunel Assay

Coronal sections (12 µm) of the brain collected at intervals of 50 µm around bregma -0.3 from animals at 1 and 4 weeks (n = 5 animals/timepoint) after surgery were examined for terminal deoxynucleotidyltransferase (TdT)-mediated deoxyuridine triphosphate (dUTP)-rhodamine nick end-labeling (TUNEL) (Roche Bioscience, Palo Alto, CA) as described previously (Hains et al., 2003). Negative controls were incubated in the absence of TdT enzyme. As a positive control, some tissue was preincubated in DNase I (1 µl/ml in 50 mM Tris-HCl, 10 mM MgCl₂, 1 mg/ml bovine serum albumin [BSA]) before TUNEL dUTP-fluorescein detection, to induce DNA nicks. Slides were mounted with Aqua-polymount (Polysciences, Warrington, PA).

Physical Disector Counting Method and Image Analysis

Stereologic counting methods were used to obtain an accurate estimate of the number of FG-labeled backfilled neurons after DC lesion (n = 5 animals/timepoint, n = 20 animals total) (Smolen et al., 1983; Mori et al., 1997; Kwon et al., 2002, Hains et al., 2003). Serial coronal sections (20 µm) were made through the M1 cortex, and every tenth section was saved and digitized spanning bregma -2.0 to 2.0. Only cells that displayed prominent nuclear profiles were scored. The total number of FG-positive cell profiles (N_{total}) was estimated by the following formula:

$$N_{\text{total}} = \frac{(t \cdot f) \cdot \sum_{i=1}^n Q_i}{t}$$

where section thickness $t = 20$ µm, and $f = 10$. The calculated distance from one disector to the next was 200 µm. Q_i = counted cell profiles in the uniformly sampled disectors (crude number); $n = 10$ (number of equidistant sections used in the analysis). The number of cells in each of the 1 to i th-sampled sections was used to calculate the coefficient of error (Gundersen et al., 1988; Abusaad et al., 1999). The percentage of co-positive FG and TUNEL cells was determined by overlaying FG and TUNEL channels for three sections per animal at bregma -0.3, where FG density was highest. Sections 12 µm thick were collected among 20-µm sections for this analysis; 8-µm-thick sections were cut to maintain 20-µm disector separation. Differences among control and experimental groups were evaluated by unpaired t -test and Wilcoxon rank-sum for two independent samples at a 95% confidence interval. All values displayed are means $\pm$ SD.

Open-Field Locomotor Testing

Behavioral analysis was performed on the SCI+FG+ OEC group (n = 6) and the SCI+FG +DMEM group (n = 6). Preoperative testing began 2 days before injury and was performed weekly for 5 weeks after surgery. Locomotor function was recorded by a blinded observer using the Basso, Beattie, and Bresnahan (BBB) locomotor rating scale (Basso et al., 1995) to ensure reliability of hind limb somatosensory testing and to assess treatment outcome. The

Mann–Whitney U-test was performed for between group comparisons for BBB analysis. Values displayed are means $\pm$ SD.

ELISA Measurements

At 1 week of survival, rats ($n = 7$ in each group, 2 groups, for a total of 14 animals) were anesthetized with an overdose of pentobarbital (75 mg/kg, i.p.) and decapitated for biochemical experiments. Spinal cords were taken out within 30–60 s after decapitation. The transplanted lesion at the T9 segment was then cut and weighed. Tissue was homogenized in 150 μ l of ice-cold lysis buffer (137 mM NaCl, 20 mM Tris-HCl; pH 8.0, 1% Nonidet P-40 (NP-40), 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μ g/ml aprotinin, 1 μ g/ml leupeptin, and 0.5 mM sodium vanadate), using a tube pestle (Fisher Scientific, Pittsburgh, PA). The sample was then centrifuged at 3,000g for 30 min at 4°C. All samples were used within 1 week after freezing. Because concentrations of neurotrophic factors are low in the spinal cord, the supernatant was pooled from 7 animals in each of two groups and stored at -80°C . Samples were run in triplicate. The supernatants were used for NGF, NT-3 and BDNF quantification (Johnson et al., 2000). The NGF E-max immunoassay system (Promega, Madison, WI) demonstrates very low cross-reactivity with structurally related growth factors [recombinant human BDNF (rhBDNF), neurotrophin 3 (rhNT3), and neurotrophin 4 (rhNT4)] at concentrations as high as 10 ng/ml, as indicated by the manufacturer (Promega). The BDNF E-max immunoassay system (Promega) shows less than 3% cross-reactivity to NGF, NT-3, and NT-4 at concentrations as high as 100 ng/ml. The NT-3 E-max immunoassay system (Promega) offers less than 3% cross-reactivity to related neurotrophic factors at 10 ng/ml. Absorbance values of standards and samples were corrected by subtraction of the background value to correct for absorbance due to nonspecific binding. The absorbance of samples was measured at 450 nm. Values displayed are means $\pm$ SEM. Statistical comparisons were made using Student's *t*-test between the SCI+FG+OEC and the SCI+FG+DMEM groups.

RESULTS

Transplanted OECs Integrate into the Spinal Cord Transection Site

In the cell suspensions used for transplantation, more than 95% of cells were p75⁺ and S-100⁺. The transplanted GFP-OECs were easily identified within the lesion zone by their green fluorescence at both 1 week (Fig. 1A) and 4 weeks (Fig. 1B) after transplantation. At 1 week, the OECs were largely confined to the lesion zone and showed variable degrees of process outgrowth (Fig. 1A, inset). At 4 weeks, the OECs were also largely confined to the lesion zone, but a number of cells extended outside of the lesion (Fig. 1B). The inset in Figure 1B shows the presence of FG in the spinal cord. At 4 weeks, some OECs aligned longitudinally with axons as shown in the inset in Figure 1C, where OECs are seen to surround NF-stained axons. Immunostaining for AnkG and Caspr define nodal and paranodal regions (Rios et al., 2000), respectively, between internodal regions formed by the transplanted OECs. Axonal alignment and nodal formation was not observed at 1 week post-transplantation.

TUNEL Staining of FG-Backfilled Cortical Neurons Shows Apoptosis of Cortical Neurons 1 Week After Dorsal Spinal Cord Transection

Bilateral transection of the dorsal corticospinal tract (dCST) and FG injection at T9 results in robust retrograde labeling of the cell bodies of these axons within the primary motor cortex (M1) (Fig. 2C), which spans from approximately bregma 1.6 through -1.6 . In the coronal plane, the anatomical location of labeled cells was within a region lateral to the midline longitudinal fissure and medial to the forelimb region of the primary sensory cortex (S1) (boxed area in Fig. 2A; modified from Paxino and Watson, 1998) and within cortical layer V. In the sagittal plane, the anterior distribution of FG-labeled cells extended to the rostral margin of the anterior commissure and extended caudally to the posterior margin of the optic chiasm. Labeled cells

displayed pyramidal-shaped somata (Fig. 2C; inset), which give rise to a singular apical dendrite and smaller basal dendrites. In the rostrocaudal dimension, the density of backfilled CST neurons was highest at approximately bregma -0.3 .

At 1 week after SCI, TUNEL-positive cells were observed within layer V of M1 and co-localized with Hoechst 33342 nuclear stain within FG backfilled pyramidal neurons (Fig. 2B–D, arrowheads). Not all FG-positive cells were TUNEL-positive (Fig. 2B–D, arrows).

TUNEL-Positive Neurons in M1 Are Reduced After OEC Transplantation

Triple labeling with Hoechst, FG, and TUNEL identified putatively axotomized pyramidal cells in the SCI group, with and without spinal OEC transplantation. The neuronal subset undergoing apoptosis was readily identified in 1-week tissue. Within these cells, nuclear morphologies of both apoptotic and surrounding normal neurons and glia could be identified (Fig. 3A,B). Hoechst staining was observed in all cells and uniformly filled the spherical nuclear compartment. In a proportion of cells undergoing apoptosis, Hoechst staining revealed abnormal morphology, including the formation of chromatin condensates within a less-distinct nucleus (Hains et al., 2003). Merging of Hoechst, FG, and TUNEL staining (Fig. 3A,B) showed that irregular nuclear fragments identified by Hoechst staining overlap securely with FG- and TUNEL-positive cells, permitting the confident designation of an apoptotic status. The nuclear morphology of TUNEL-positive neurons was identical to that seen in our earlier paper (Hains et al., 2003) (Fig 3A, insets).

Apoptotic (FG- and TUNEL-positive cells) pyramidal neurons were observed both after SCI +FG+DMEM (Fig. 3A, arrowheads) and in the SCI+FG+OEC group (Fig. 3B, arrowheads), but the number of apoptotic neurons was significantly reduced in the SCI+FG+OEC group (Fig. 3C). At 1 week after injury, the number of apoptotic neurons was significantly ($P < 0.05$) reduced by 45% (16.4 ± 6.40 vs. 36.1 ± 5.69) in the OEC transplant group compared with the SCI+FG+DMEM group (Fig. 3C). At 4 weeks, both the SCI+FG+DMEM and SCI+FG+OEC groups showed apoptotic activity comparable to sham controls (0.98 ± 0.81 and 0.75 ± 0.56) (Fig. 3C).

OEC Transplantation Reduces Corticospinal Neuronal Loss

At 1 week after SCI, the number of FG-positive neurons was significantly ($P < 0.05$) higher in the SCI+FG+OEC transplant group. Figure 4A,B shows corresponding sections through bregma -0.3 of both groups. Quantification of the number of FG-positive neurons at 1 week (Fig. 4C) and 4 weeks (Fig. 4D) at various antero-posterior positions in M1 indicates increased neuronal survival at both timepoints for the SCI+FG+OEC transplant group. Stereological analysis indicates that the difference in total neuronal counts between SCI+FG+DMEM and SCI+FG+OEC groups is $28.0 \pm 3.76\%$ at 1 week, and $51.1 \pm 4.58\%$ at 4 weeks (Fig. 4C,D). This is a significant difference ($P < 0.05$), and shows that neuronal loss is greater in the SCI+FG+DMEM group. Definite integral area analysis shows that loss of pyramidal neurons continued from 1 week to 4 weeks in the SCI+FG+OEC, but the loss was less than in the SCI+FG+DMEM group. OEC transplantation caused a sparing of $24.3 \pm 1.96\%$ and $46.3 \pm 3.04\%$ of the total number of FG-positive pyramidal cells, at 1 and 4 weeks, respectively, compared with the SCI+FG+DMEM group (Fig. 4E). This amount of sparing is significant ($P < 0.05$) in the transplant group.

Elevation in BDNF at the OEC Transplantation Site and Improved Locomotor Activity

A number of neurotrophins have been suggested to enhance neuronal survival and axonal regeneration after SCI (Boruch et al., 2001; Woodhall et al., 2001). NGF, NT-3, and BDNF were assayed from the injured spinal cord segments with and without OEC transplantation.

BDNF was significantly elevated in the spinal segment of the OEC transplantation site 1 week after SCI ($P < 0.05$). No difference was detected in NGF and NT-3 levels (Fig. 4F).

To ensure further that our lesion model and transplantation protocol conformed to previous use of this model (Sasaki et al., 2004), open-field locomotor testing (BBB) was carried out on the SCI+FG+DMEM and the SCI+FG+OEC transplant groups. As reported previously for this model system (Sasaki et al., 2004), OEC transplantation resulted in a significant improvement in open-field locomotor activity in the OEC transplant group (Fig. 4G). At 5 weeks post-transplantation BBB scores were 9.0 ± 1.0 in the SCI+FG+DMEM group and 15.5 ± 1.5 in the SCI+FG+OEC group ($P < 0.01$).

DISCUSSION

Corticospinal tract neurons (CST) undergo apoptosis after transection of their spinal axonal projections as observed at 1 week post-injury (Hains et al., 2003). In the present study we asked whether transplantation of OECs into the dorsal transected spinal cord reduces apoptosis and cell death in corticospinal neurons of M1. The results demonstrate a significant reduction of apoptotic M1 neurons at 1 week post-transplantation and a larger number of surviving CST neurons at 4 weeks post-injury after OEC transplantation. Our findings demonstrate that a larger available pool of cortical neurons is present after OEC transplantation at the spinal cord injury site, indicating a neuroprotective effect subsequent to OEC transplantation on long tract projecting axons.

We previously characterized apoptotic cell death of CST neurons after transection of their axons in the spinal cord by TUNEL assay and by identification of internucleosomal DNA fragmentation (Hains et al., 2003). In the current study, the TUNEL method was used due to its high sensitivity for detecting apoptosis in a variety of tissues (Gavrieli et al., 1992; Migheli et al., 1995). While we cannot rule out the possibility of necrotic cell death of some CST neurons, the cell counts of FG-positive cells clearly indicate a preservation of axotomized CST neurons in the spinal cord after OEC transplantation.

OECs are known to express a number of neurotrophic factors, including NGF and BDNF, but not NT-3 (Boruch et al., 2001; Woodhall et al., 2001), and several studies suggest that exposure to neurotrophins can limit death of axotomized neurons in the CNS after injury (for review see Giehl, 2001). One possibility to account for the neuroprotective effect of OEC transplantation is delivery of neurotrophic factors to the injured axons by the engrafted OECs. We found detectable increases in BDNF, but not in NGF and NT-3, 1 week after cell transplantation, suggesting that OECs could be secreting trophic substances and/or may facilitate endogenous neurotrophic secretion. Consistent with this conclusion, paracrine BDNF support has been shown to be necessary for survival of CST neurons after proximal axotomy (Giehl and Tetzlaff, 1996; Schutte et al., 2000; Giehl et al., 2001), and BDNF promotes sprouting of CST axons after axotomy in the spinal cord (Namiki et al., 2000; Hiebert et al., 2002; Zhou and Shine, 2003). It is important to note that endogenous Schwann cells can invade the spinal cord after OEC transplantation (Takami et al., 2002; Boyd et al., 2004; Ramer et al., 2004), so changes in neurotrophic factor availabilities could possibly result from endogenous Schwann cells as well. Moreover, possible Schwann cell contamination in OEC preparations has been discussed (see Boyd et al., 2005 for an overview). While we cannot rule out minor contamination by Schwann cells in our OEC preparation, our acute cell preparation consists of nonexpanded cells and over 95% of the cells are p75⁺ and S-100⁺ (Akiyama et al., 2004; Sasaki et al., 2004). This large number of p75⁺ and S-100⁺ cells from acutely dissociated outer nerve layer of the olfactory bulb, which is enriched in p75⁺ OECs (Au et al., 2002), indicates that Schwann cell contamination, if present, is minor.

The injured spinal cord can spontaneously form new intraspinal circuits in the rat; propriospinal neurons can arborize on lumbar motor neurons after dorsal hemisection (Bareyre et al., 2004). This new intraspinal circuit relays corticospinal information to motor targets below the lesion level. One possibility to account for the improved locomotor function after OEC transplantation is that the increased survival of corticospinal neurons by OECs provides for enhanced intraspinal circuit construction. While the focus of this study was on rescue of CST neurons by intraspinal transplantation of OECs, it is possible that neurons from other descending systems (e.g., rubrospinal) may also be rescued by the transplantation procedure. Axonal die-back can occur after CST transection that could potentially influence the survival of the parent neuron. We are not certain if the OEC transplants modulate axonal retraction.

Several groups have demonstrated functional recovery after OEC transplantation in the injured spinal cord (Ramon-Cueto et al., 2000; Plant et al., 2003; Sasaki et al., 2004; Garcia-alias et al., 2005). While the precise mechanism of this functional recovery is not fully understood, several mechanisms including long tract regeneration (Li et al., 1997; Ramon-Cueto et al., 2000), axonal sparing (Plant et al., 2003), sprouting and plasticity associated with novel polysynaptic pathways (Keyvan-Fouladi et al., 2002; Bareyre et al., 2004), recruitment of endogenous Schwann cells (Takami et al., 2002; Boyd et al., 2004; Ramer et al., 2004) and remyelination (Franklin et al., 1996; Sasaki et al., 2004), have been suggested to contribute to improvement in functional outcome in SCI after OEC transplantation. The present study demonstrates enhanced survival of CST neurons after dorsal spinal cord transection and OEC transplantation, suggesting a potentially additional role of neuroprotection by OECs on descending motor control systems.

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Fig. 1.

Sagittal frozen sections through the injury and GFP-OEC transplantation site 1 week after injury (**A**). Inset shows variable degrees of process outgrowth of transplanted GFP-OECs in the spinal cord. Four weeks after transplantation (**B**), transplanted cells survive and are distributed extensively within the transplantation site, as well as to a limited extent beyond the lesion site. Inset in B shows the presence of FG in the spinal cord. **C**: AnkyrinG (red) and Caspr (blue) staining demonstrate nodal and paranodal regions, respectively, associated with some transplanted OECs (green). In this section, 34 pairs of paranodal Caspr stained regions were identified. Twenty-five of the 34-paranodal Caspr regions (74%) were associated with green fluorescence. Nineteen of these 25 Caspr-stained regions had AnkG immunofluorescence at the putative nodal site, indicating that most identified nodal–paranodal regions within the lesion were associated with the transplanted OECs. Inset in C shows NF immunostaining that reveals intimate relationship between axons and GFP expressing OECs at 4 weeks. Scale bars = 500 μ m in A, B; 50 μ m in inset in A; 5 μ m in C; 10 μ m in inset in C.

Fig. 2. Schematic representation (**A**) of a coronal atlas section of rodent brain corresponding to bregma -0.3 (Modified from Paxino and Watson, 1998), illustrating the location of Fluorogold (FG)-positive backfilled axotomized corticospinal pyramidal neurons in primary motor cortex (M1), 1 week after injury. Hoechst 33342-labeled nuclei (**B**) correspond to FG-positive neurons (**C**, inset higher magnification) within layer V. TUNEL labeling to identify neurons with DNA strand breaks (**D**) reveals that a fraction of FG-positive corticospinal neurons were undergoing apoptotic cell death (arrowheads), while some FG-positive neurons were not identified by the TUNEL assay (arrows with tails). TUNEL labeling was restricted to the nuclear compartment of cells. Scale bar = $125\ \mu\text{m}$.

Fig. 3.

Hoechst 33342, Fluorogold, and TUNEL triple labeling of corticospinal neurons 1 week after injury. Hoechst staining of non-TUNEL-positive (arrows with tails) and TUNEL-positive (arrowheads) neurons with corresponding FG-backfilling are shown. In SCI+FG+OEC animals (**B**), fewer TUNEL-positive FG-backfilled neurons are observed compared with SCI+FG+DMEM (**A**). Insets in A show two TUNEL-positive neurons exhibiting nuclear compartmentalization and formation of nucleosomes, hallmarks of apoptosis. Quantitation of neurons that are both TUNEL- and FG-positive (**C**) reveals that OEC transplantation significantly ($*P < 0.05$) reduces apoptotic cell death at 1 week. No evidence of death was observed at any other timepoint. Scale bars = 125 μ m in A,B; 20 μ m in inset in A.

Fig. 4.

Density of FG-positive corticospinal neurons at bregma -0.3 at 1 week after axotomy is greater in SCI+FG+OEC animals (**B**), when compared with SCI+FG+DMEM animals (**A**). Serial coronal sections spanning bregma -2.0 through 2.0 from SCI+FG+DMEM and SCI+FG+OEC animals at both 1 (**C**) and 4 weeks (**D**) shows that in SCI+FG+OEC animals (filled circles), FG-positive neuron density is higher at both 1 and 4 weeks, when compared with SCI+FG+DMEM animals (open circles). From 1 to 4 weeks, both groups demonstrate a decrease in FG-positive backfilled neuron density, but SCI+FG+OEC animals showed a reduced amount of loss by integral analysis. Quantitation of the mean number of cells at bregma -0.3 reveals that OEC transplantation significantly ($*P < 0.05$) attenuated cell loss after injury (**E**). ELISA analysis of NGF, NT-3, and BDNF at the site of transplantation in SCI+FG+OEC and SCI+FG+DMEM animals at 1 week are shown. In comparison to controls, a significant ($*P < 0.05$) increase in BDNF is measured; no significant changes in NGF and NT-3 are detected (**F**). Open-field locomotor scores for SCI+FG+OEC ($n = 6$) and SCI+FG+DMEM ($n = 6$) groups tested 1 week before and for 5 weeks after transplantation (**G**). All animals exhibited a gradual

improvement in hindlimb locomotor function during the 5-week recovery period. The Mann–Whitney U-test indicated that the open-field locomotor scores at 3, 4, and 5 weeks after injury and SCI+FG+OEC group are significantly higher than the SCI+FG+DMEM (* $P < 0.05$, ** $P < 0.01$); Calibration in B corresponds to 125 μm .