Brain Advance Access published December 3, 2007

doi:10.1093/brain/awm267 *Brain* (2007) Page 1 of 13

OEG implantation and step training enhance hindlimb-stepping ability in adult spinal transected rats

Marc D. Kubasak,[1] Devin L. Jindrich,[1,2,*] Hui Zhong,[2] Aya Takeoka,[1] Kimberly C. McFarland,[1] Cintia Muñoz-Quiles,[3] Roland R. Roy,[2] V. Reggie Edgerton,[1,2] Almudena Ramón-Cueto[3] and Patricia E. Phelps[1,2]

[1]Department of Physiological Science, UCLA, Los Angeles, CA, [2]Brain Research Institute, UCLA, Los Angeles, CA, USA and [3]Institute of Biomedicine, CSIC, Valencia, Spain

*Present address: Department of Kinesiology, 551 E. Orange St, PEBE 107B, Tempe, AZ 85287-0404, USA

Correspondence to: Dr Patricia E. Phelps, Department of Physiological Science, UCLA, Box 951606, Los Angeles, CA 90095-1606, USA.
E-mail: pphelps@physci.ucla.edu

Numerous treatment strategies for spinal cord injury seek to maximize recovery of function and two strategies that show substantial promise are olfactory bulb-derived olfactory ensheathing glia (OEG) transplantation and treadmill step training. In this study we re-examined the issue of the effectiveness of OEG implantation but used objective, quantitative measures of motor performance to test if there is a complementary effect of long-term step training and olfactory bulb-derived OEG implantation. We studied complete mid-thoracic spinal cord transected adult female rats and compared four experimental groups: media-untrained, media-trained, OEG-untrained and OEG-trained. To assess the extent of hindlimb locomotor recovery at 4 and 7 months post-transection we used three quantitative measures of stepping ability: plantar stepping performance until failure, joint movement shape and movement frequency compared to sham controls. OEG transplantation alone significantly increased the number of plantar steps performed at 7 months post-transection, while training alone had no effect at either time point. Only OEG-injected rats plantar placed their hindpaws for more than two steps by the 7-month endpoint of the study. OEG transplantation combined with training resulted in the highest percentage of spinal rats per group that plantar stepped, and was the only group to significantly improve its stepping abilities between the 4- and 7-month evaluations. Additionally, OEG transplantation promoted tissue sparing at the transection site, regeneration of noradrenergic axons and serotonergic axons spanning the injury site. Interestingly, the caudal stump of media- and OEG-injected rats contained a similar density of serotonergic axons and occasional serotonin-labelled interneurons. These data demonstrate that olfactory bulb-derived OEG transplantation improves hindlimb stepping in paraplegic rats and further suggest that task-specific training may enhance this OEG effect.

Keywords: locomotion; regeneration; spinal cord injury; rehabilitation; olfactory ensheathing glia

Abbreviations: 5-HT = serotonin; DBH = Dopamine Beta Hydroxylase; FFT = Fast Fourier Transform; GFAP = Glial Fibrillary Acidic Protein; IFFT = integrated FFT; OEG = olfactory ensheathing glia; PWR = peak wavelet resemblance; TBS = 0.1 M Tris buffer with 1.4% NaCl and 0.1% bovine serum albumin

Received September 11, 2007. Revised October 7, 2007. Accepted October 8, 2007

OEG transplantation reportedly facilitates axon regeneration, reduces tissue loss and improves motor performance in different models of spinal cord injury (Ramón-Cueto and Nieto-Sampedro, 1994; Li *et al.*, 1997; Imaizumi *et al.*, 1998; Kato *et al.*, 2000; Li *et al.*, 2003; García-Alías *et al.*, 2004; Fouad *et al.*, 2005). Strikingly, several reports demonstrate improved motor function and/or axonal regeneration after OEG transplantation in complete spinal cord transected adult animals, the most stringent model of spinal cord injury (Ramón-Cueto *et al.*, 1998, 2000; Lu *et al.*, 2001, 2002; López-Vales *et al.*, 2006). Here we re-examine the issue of the efficacy of olfactory bulb-derived OEG transplantation and test whether treadmill step training can enhance the long-term (7 months) therapeutic effects of OEG transplantation in adult spinal transected rats.

Treadmill step training alone can improve coordinated motor function in completely transected adult cats (Lovely *et al.*, 1986, Barbeau and Rossignol, 1987, de Leon *et al.*, 1998) and neonatal rats (Kubasak *et al.*, 2005) in the absence of any demonstrated axonal regeneration across the lesion. Similar improvement of motor function associated with treadmill training occurs in humans with severe, but incomplete spinal cord injury (Barbeau and Rossignol, 1994; Harkema *et al.*, 1997; Wernig *et al.*, 2000). Following a complete transection model of spinal cord injury, hindlimb motor activity is thought to occur by activating spinal circuits that can interpret complex proprioceptive input (Dietz, 2003; Edgerton *et al.*, 2004) and stimulate central pattern generation (Grillner *et al.*, 2005). The training-induced reorganization of these circuits improves motor behaviour and can reduce the inhibitory potential of the GABAergic (Tillakaratne *et al.*, 2002) and glycinergic (de Leon *et al.*, 1999) systems associated with somatic motor neurons. Additionally, serotonergic and noradrenergic fibres are associated with fine motor control during stepping and local administration of serotonergic and noradrenergic agonists improves stepping performance while antagonists block the activity of the central pattern generators (Barbeau and Rossignol, 1991; Barbeau *et al.*, 1993; Veasey *et al.*, 1995; Ribotta *et al.*, 2000; Giroux *et al.*, 2001; Guertin, 2004). Furthermore, step training modifies motor neuron responses to segmental and afferent stimulation (Côte *et al.*, 2003; Côte and Gossard, 2004; Petruska *et al.*, 2007). Thus, electrophysiological changes in the spinal circuitry for locomotion occur following step training in completely transected animals.

We hypothesized that OEG implantation would enhance recovery of motor function after a complete transection possibly by promoting the regeneration of supraspinal axons into the distal spinal cord stump (Ramón-Cueto *et al.*, 1998, 2000; Lu *et al.*, 2001, 2002; López-Vales *et al.*, 2006). We further reasoned that motor training would enhance the effects of OEG treatment perhaps by reorganizing the spinal sensorimotor circuits (Edgerton *et al.*, 2004) and/or facilitating the formation of functional neuronal connections across the lesion. Our results show that transplantation of olfactory bulb-derived OEG is sufficient to promote hindlimb plantar stepping after a complete transection in adult rats and suggest that extensive training can augment this recovery.

Materials and Methods

OEG cell cultures

Methods were adopted from those of Ramón-Cueto *et al.* (2000). Wistar Hannover rats (8–10 weeks old) were purchased from Harlan Laboratories (Indianapolis, IN). OEG were obtained from the first two layers of the olfactory bulbs and immunopurified with the p75-receptor antibody (Supplemental Fig. 1; Chandler *et al.*, 1984; clone 192, 1:5). Prior to transplantation, cells were resuspended at a concentration of 100 000 cells per µl of serum-free Dulbecco's Modified Eagle's Medium.

Surgery and spinal cord injections

Female Wistar Hannover rats were acclimated to the treadmill and body harness for 2 weeks before surgery. All rats were 10–12 weeks old at the time of surgery. All procedures followed the NIH guidelines and were approved by the Chancellor's Animal Research Committee at UCLA. Animals were anaesthetized with 2% isoflurane gas mixed with oxygen. A partial laminectomy of the T8 and T9 vertebrae was performed and the dorsal dura was opened with an 'H' cut to expose the spinal cord. The spinal cord then was completely transected at approximately spinal cord level T9 with micro-scissors, leaving the ventral and lateral dura intact. A probe was passed through the transection site and the spinal cord stumps were gently lifted as two surgeons confirmed a complete transection. We used a stereotactic apparatus to inject either OEG suspended in Dulbecco's Modified Eagle's Medium or Dulbecco's Medium alone. Injections were made with sterile glass needles (100 µm diameter) 1 mm from the transection site. Four injections of ~50 000 cells each were made into each spinal cord stump for a total of 400 000 cells per rat. The injection sites were identical to those described by Ramón-Cueto *et al.* (2000). The paravertebral muscles and fascia were sutured using 4-0 Chromic gut and the skin incision closed with 4-0 Ethilon suture. The rats recovered in an incubator (37°C) and received Lactated Ringers (5 cc, s.c.) to prevent dehydration. Baytril (0.2 cc, i.m., b.i.d., enrofloxacin, Bayer HealthCare LLC), a general antibiotic, and Buprenex (0.05 mg/kg, s.c., b.i.d., buprenorphine hydrochloride, Reckitt Benckiser Healthcare (UK) Limited), an analgesic, were administered during the first 2 days of recovery. Bladder expressions were performed three times a day for three days and then twice daily, at 12 h intervals, thereafter. Rats were housed individually in a room maintained at 24 ± 1°C with 40% humidity and a 12:12 h light:dark cycle. Sham rats received a partial laminectomy of the T8 and T9 vertebrae and the dura was opened, but no transection was performed. General-care procedures for spinal cord injured animals are published (Roy *et al.*, 1992).

Step training

To acclimate the animals to the training apparatus, the first week began with 5 min of step training and the training time was increased by 5 min each week for the first month. For the next 5 months, rats were trained 20 min/day, 5 days/week. The rats were suspended in a body harness over the treadmill in a semi-erect position to facilitate bipedal stepping. We attached robotic arms (Phantom 1.0, SensAble Technologies, Inc., Woburn, MA) to the rat's ankles and moved them manually in a pattern designed to mimic bipedal stepping of intact (sham) animals. During step training we took special care to keep the toes extended and to ensure contact on the treadmill with the footpad during the stance phase. Interlimb coordination was maintained throughout training to maximize the response. Each trained spinal rat received a total of more than 50 h of manual step training.

Stepping ability was evaluated at 4 and 7 months post-surgery at a treadmill speed of 13 cm/s and 85% body weight support (i.e. the animal's hindlimbs supported 15% body weight). Evaluations were videotaped and examined with a motion analysis program (Motus, Peak Performance Technologies, Englewood, CO). For the final evaluation, the hindlimbs were shaved, reflective markers were

Table 1 Stepping performance and the presence of serotonergic and noradrenergic fibres

Experimental group	Plantar steps at 7 months (no.)	Amount of tissue block examined for 5-HT (%)	5-HT-positive fiber density (fibres/mm^3)	Amount of tissue block examined for NA (%)	DBH-positive fiber density (fibres/mm^3)
Media-untrained	0	50	5	50	3
Media-untrained	0	50	28	50	1
Media-untrained	0	50	19	50	0
Media-trained	0	45	19	45	1
Media-trained	0	49	25	49	0
Media-trained	2	50	14	50	0
Media mean±SEM			18±3		1±0
OEG-untrained	0	45	23	45	34
OEG-untrained	12	44	0	44	1
OEG-untrained	26	49	5	n/a[a]	n/a
OEG-untrained	8	n/a[a]	n/a	35	11
OEG-trained	0	40	2	40	2
OEG-trained	14	29	38	18	37
OEG-trained	21	46	20	46	1
OEG mean ± SEM			15 ± 6		14 ± 7*

n/a = not available.
*$P = 0.03$.
[a]Two different OEG-untrained animals were analysed for 5-HT- and DBH-positive fibres, respectively.

placed on the foot, ankle, knee, hip and iliac crest, and the markers were tracked in two camera views to yield three-dimensional coordinates using direct linear transform. Internal ankle, knee and hip joint angles were calculated from the markers spanning each joint and used to perform kinematic analyses. Plantar steps were counted for both legs by three evaluators blind to the experimental groups using the videotaped evaluations recorded 4 and 7 months post-surgery. The total number of plantar steps was averaged within each treatment group.

Tissue preparation and immunocytochemical procedures

We processed spinal cords from all experimental groups with an identical protocol. Rats were anaesthetized with Ketamine (0.9 µl/g) and Anased (0.5 µl/g) and perfused intracardially with 4% paraformaldehyde in 0.12 M phosphate buffer. Animals were post-fixed overnight and washed in buffer before the spinal cords were dissected. Once removed, the spinal cords were washed thoroughly in buffer. Prior to embedding, the injury sites were photographed on a black background. Spinal cords were cryoprotected in 30% sucrose, blocked, embedded in Tissue-Tek Optimal Cutting Temperature, and stored at −80°C. Using a cryostat, 25-µm-thick sagittal sections of the block including the transection site were cut and mounted on a series of 20 slides so that each slide contained every 20th section. After an average of 6–7 sections were mounted per slide, they were stored in buffer with sodium azide at 4°C until processing.

Sections were washed with 0.1 M Tris buffer containing 1.4% NaCl and 0.1% bovine albumin (TBS), followed by a 30-min presoak in 0.3% H_2O_2 and 0.1% sodium azide. After a 0.8% Triton presoak for 15 min, sections were incubated in 1.5% normal horse serum with 0.1% Triton and an additional blocking step to reduce non-specific biotin/avidin binding (Vector Laboratories; Burlingame, CA). Sections were placed into primary antibody solution overnight to localize the distribution of Glial Fibrillary Acidic Protein (GFAP)-positive astrocytes (Mouse Anti-GFAP, 4A11, 1:1000, BD Biosciences, San Jose, CA) or Dopamine Beta Hydroxylase (Mouse Anti-Rat DBH, 1:1000, Chemicon, Temecula, CA) to identify coeruleospinal noradrenergic axons. Subsequently, sections were rinsed in TBS before incubation in biotinylated rat-absorbed goat anti-mouse IgG (diluted 1:200 in TBS, Vector Laboratories) for 1 h and followed by TBS containing the avidin–biotin complex (1:100, Elite Standard Vectastain ABC Kit; Vector Laboratories). Following an acetate buffer rinse, diaminobenzidine was intensified with nickel-glucose oxidase. After a final acetate buffer rinse, sections were dehydrated and coverslipped.

To identify 5-HT-positive fibres, we used methods identical to those detailed above except for the substitution of the goat anti-serotonin antibody (1:20 000, ImmunoStar, Hudson, WI), biotinylated anti-goat IgG (1:200), and the Elite Goat IgG Vectastain ABC Kit. For double-labelling procedures 5-HT or DBH was visualized using diaminobenzidine amplified with nickel-glucose oxidase producing a black reaction product. Then sections were rinsed, processed for GFAP, and visualized with diaminobenzidine containing 0.02 M imidazole that produced a contrasting amber-brown product. We used GFAP-immunocytochemistry to determine if the spinal cord transections were complete. The GFAP-negative area showed a complete separation of rostral and caudal spinal cord stumps in all transected rats (Supplemental Figs. 2 and 3).

Morphological analyses

Animals selected for statistical analyses represented a range of stepping abilities (Table 1). For the OEG animals we chose one of the best steppers, one of the worst, and one in between (Table 1). Most media-injected rats failed to perform any plantar steps so we chose rats from these groups randomly. In each plantar stepping category we used the animals where we had the largest samples processed for both 5-HT- and DBH-labelled immunoreactivity. We counted the number of labelled axons caudal to the transection site from three rats per antibody in each experimental group.

For each animal, we analysed between 18 and 50 sections/antibody to quantify the number of labelled axons caudal to the transection site. We used Openlab software (Improvision Inc., Lexington, MA) to identify fibres in the GFAP-negative region and counted all immunopositive axons longer than 25 µm throughout the entire GFAP-positive caudal stump of each section. Subsequently we measured the size of each tissue section (see later) to calculate the areal density. We grouped fibres into those that extend up to 250 µm beyond the caudal GFAP-interface and those coursing further distances into the caudal stump (or endogenous fibres) (Xu *et al*., 1999).

To analyse tissue sparing we examined 10–15% of the sections distributed evenly throughout the transection site from the same three animals per experimental group reported earlier. Starting with the first identifiable section, sequential sections at 175–200 µm intervals were analysed throughout the spinal cord. Analyses included tracing and measuring each tissue section and the GFAP-positive region within the block rostral and caudal to the transection. We estimated the total volume of the injury site as the GFAP-negative area between the two GFAP-positive regions (Cheng and Olson, 1995; Iannotti *et al*., 2006). Comparisons were not made between the spinal cords of transected and intact rats. Camera lucida drawings were made from representative OEG- and media-injected animals. Original drawings from a series of seven alternating 25-µm-thick sagittal sections were scanned, overlaid and merged. Fibre thickness was enhanced to improve visualization.

Kinematic analyses

We evaluated two aspects of the stepping kinematics for each rat: movement frequency and movement shape. To assess movement frequency, we used a technique based on the Fast Fourier Transform (FFT) similar to that described by Kubasak *et al*. (2005). For each joint internal angle, the power spectrum was calculated from 8 s of continuous stepping using the FFT, and the frequency at peak power was identified. The mean and SD of the frequency at peak power was calculated across all five sham animals to assess the range of frequencies associated with the peak power during restrained bipedal locomotion in sham rats. We constructed a bandpass filter centred at the mean frequency at peak power with a width of 2 SD. The FFT power spectra for all trials of each treatment group and each joint angle were passed through the filter, integrated and averaged across the ankle, knee and hip joints of each hindlimb. The resultant average value of the integrated FFT (IFFT) was used as an index of the degree that a hindlimb exhibited movement kinematics with frequencies observed with restrained bipedal treadmill locomotion in sham rats.

To evaluate movement shape, we used an analysis based on the continuous wavelet transform (Burke-Hubbard, 1998). Foot touchdown and liftoff events were used to identify individual steps in sham animal trials. Joint angle kinematics were rescaled to cycle phase using foot touchdown and liftoff events, then averaged to yield mean kinematic trajectories. Mean trajectories were averaged across animals to yield characteristic trajectories for normal bipedal locomotion at 13 cm/s. The trajectories were normalized to zero mean, shifted in phase so that the beginnings and ends were maximally close to zero, and then fitted with a 10th-order polynomial. The resulting polynomials for each joint angle trajectory were used as mother wavelets, and the continuous wavelet transforms were calculated for each joint angle using scales of 50–150% of the mean stride duration in 5% increments. At each scale, positive continuous wavelet transform coefficients indicate a positive correlation between the signal and the wavelet at that scale. The positive coefficients were summed algebraically across joint angles to yield a measure of the degree of step-like kinematics, a 'wavelet resemblance' for the entire hindlimb at each scale and time point. The mean-squared of the wavelet resemblance values at each scale was calculated and the peak wavelet resemblance (PWR) across scales was used as a measure of the degree to which the hindlimb exhibited step-like kinematics (although not necessarily at the same scale or frequency characteristic of sham rats). We compared the best stepping behaviour observed in each rat. Consequently, we selected the hindlimb in each transected rat with the best IFFT and PWR values for analysis, and normalized the IFFT and PWR values to the mean values from sham rats.

Although locomotor performance following spinal cord injury is often assessed with a BBB scale (Basso *et al*., 1995), this scale does not provide data on important features of locomotion in completely transected rats. However, due to the common use of the BBB scale we compared the essential criteria of the BBB ratings with our quantitative measurements. None of the OEG- or media-injected rats in any of the four groups generated occasional weight supported plantar steps in an open field, one of the criteria for reaching a level of 10 on the BBB scale. The assays used in this study provide quantitative assessments of the degree of coordination among limb segments during weight-bearing stepping and the rhythmicity of these cyclic movements at a controlled stepping speed. Additionally, the number of successful plantar-placed steps reflects a combination of critical features of successful locomotion, including the level of interlimb coordination and digit extension.

Statistical analyses

All statistical comparisons for this study were conducted using a resampling method (Efron and Tibshirani, 1991). Resampling or 'bootstrap' analyses use computational simulations of the null hypothesis to test statistical hypotheses, with minimal assumptions about the underlying distributions or estimators. Random samples are drawn (with replacement) from the pooled data for both conditions being investigated, and the estimator (i.e. the mean) calculated for each sample. Given the distribution of estimators from many samples, the probability that the observed difference could arise at random can be evaluated, and compared to a predetermined cutoff (i.e. the 95th percentile) for significance. Due to the highly bimodal distributions of the plantar-stepping values, these data were rank ordered prior to statistical analysis. For all comparisons, significance was set at $P < 0.05$. Custom scripts were developed to analyse the data using the Resampling Stats in MATLAB software package (Resampling Stats. Inc., Arlington, VA; MATLAB 7.0, Mathworks, Inc., Natick, MA). The scripts were validated against a standalone version of the software.

Results

Experimental design and motor-function assessments

Immediately after a complete spinal cord transection at T9, 20 adult rats received injections of olfactory bulb-derived

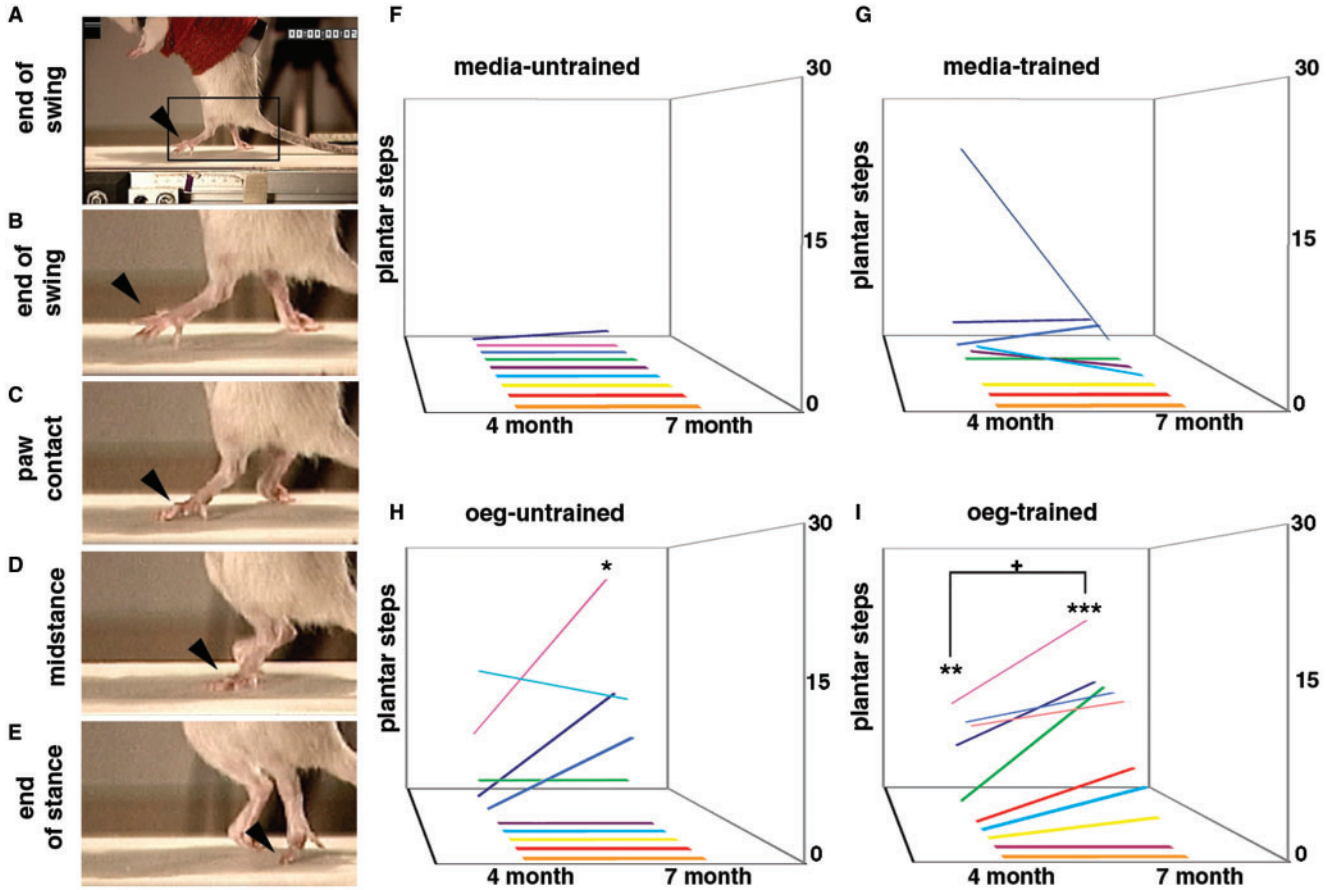

Fig. 1 OEG transplantation promotes plantar stepping in adult spinal rats and is augmented by step training. (**A**–**E**) An example of a plantar step from an OEG-untrained animal. (A, B) The plantar step shows extension of digits at the end of swing phase (arrowhead in B, enlarged from boxed area in A). (C-E) The plantar surface of the foot and toes (arrowheads) touch the treadmill at paw contact (C), and remain in contact with the treadmill through the midstance (D) and end of stance (E) phases. (**F**–**I**) Plantar step counts at 4 and 7 months post-injury for media-untrained (F, $n = 9$), media-trained (G, $n = 9$), OEG-untrained (H, $n = 10$) and OEG-trained (I, $n = 10$) rats. Significant differences in the mean number of plantar steps exist between the media-untrained and the OEG-untrained groups at 7 months ($^{*}P = 0.02$) and between the media-untrained and OEG-trained groups at 4 ($^{**}P = 0.02$) and 7 ($^{***}P \leqslant 0.001$) months. Plantar step counts are significantly different between OEG-trained animals evaluated at 4 and 7 months ($+P = 0.03$). The number of rats with improvement in plantar stepping between the 4 and 7 month tests is significantly different between the OEG-trained and the media-untrained ($P = 0.001$), media-trained ($P = 0.001$) and OEG-untrained ($P = 0.03$) groups.

OEG cells into both the rostral and caudal stumps, while 18 rats received identical injections of media alone. One month later, one-half of each group began a regimen of weight-bearing hindlimb treadmill step training. Four experimental groups are compared: media-untrained ($n = 9$), media-trained ($n = 9$), OEG-untrained ($n = 10$) and OEG-trained ($n = 10$). Eight additional rats underwent sham surgery and four were step-trained.

We used three measures of locomotor performance to assess motor recovery. The first was similar to our previous report where we counted the number of consecutive plantar-placed steps performed until failure (Lovely *et al.*, 1986). Plantar stepping reflects one of the most advanced levels of recovery following a complete spinal-cord transection as transected adult rats rarely perform plantar stepping without some effective chronic intervention. In successful plantar stepping the plantar surface of the foot contacts the treadmill belt at the beginning of stance (Fig. 1A and B), during initial paw contact (Fig. 1C), and remains on the treadmill belt throughout the stance phase (Fig. 1D and E). Step failure occurs when the animal drags its hindlimbs on the treadmill. We analysed the mean number of plantar steps per group and the change in the number of plantar steps made between the 4- and 7-month evaluations.

Secondly, we quantified hindlimb kinematics of the hip, knee and ankle during bipedal stepping. Compared to sham rats, media-untrained and media-trained animals had shorter steps, smaller excursions at all joints, and their distal joints dragged during the swing phase (Fig. 2A). These patterns contrast with many OEG-untrained and trained rats that performed more consistent stepping with

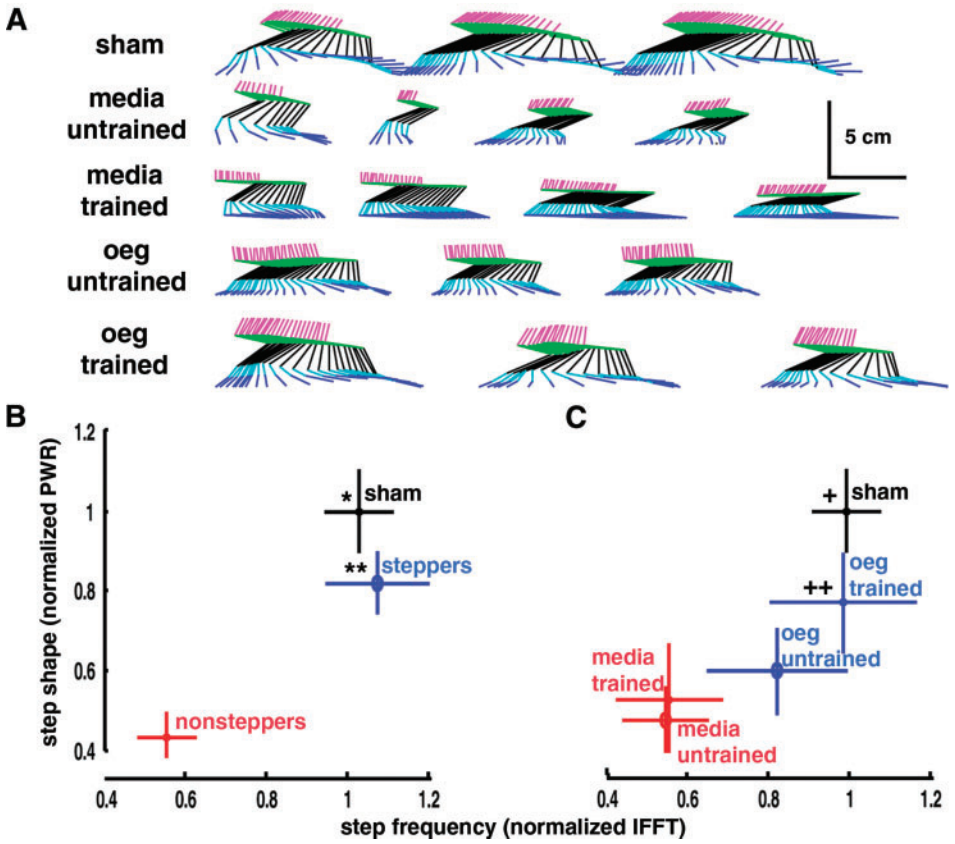

Fig. 2 The combination of OEG transplantation and step training improves stepping kinematics. (**A**) Stepping kinematics from representative trials that exhibit the IFFT and PWR values closest to the mean values for each of the five groups. Segments run from the iliac crest to the proximal femur (magenta), femur to the knee (green), knee to the ankle (black), ankle to the distal metatarsals (light blue) and distal metatarsals to the end of the toes (dark blue). Figures represent projection of kinematics onto the sagittal plane. (**B**, **C**) Step shape (represented by PWR values) is plotted against step frequency (IFFT values) and data are normalized to mean values for shams and represent the group means (±SEM). (B) Normalized IFFT and PWR scores for animals that took at least one plantar-placed step (steppers), animals that did not step (nonsteppers), and sham rats. IFFT and PWR scores of the nonstepping animals are significantly different from the sham (IFFT $P < 0.01$, PWR $P < 0.01$) and stepping (IFFT $P < 0.01$, PWR $P < 0.01$) animals. Stepping animals are not significantly different from the shams (IFFT $P = 0.43$, PWR $P = 0.11$). (C) IFFT and PWR scores of the media-untrained and media-trained animals are significantly different from sham (IFFT $P = 0.01$, PWR $P < 0.01$ for media-untrained; IFFT $P = 0.01$, PWR $P = 0.02$ for media-trained). OEG-untrained rats do not differ from shams in IFFT ($P = 0.24$) but show significantly lower PWR scores ($P = 0.01$). OEG-trained animals are not significantly different from shams (IFFT $P = 0.48$, PWR $P = 0.11$).

larger joint excursions, particularly at the ankle joint, and substantial foot clearance during swing (Fig. 2A). To assess the consistency of stepping across the treatment groups, we developed a method based on filtering and integrating the power spectrum of the internal joint angles calculated from the Fast Fourier Transformation (IFFT, see 'Methods' section).

Thirdly, we used a method based on the continuous wavelet transform to evaluate the extent to which the shape of the hindlimb-joint movements during stepping resembled those of sham rats. Using a measure termed the PWR, we compared the patterns of stepping kinematics among all groups. IFFT and PWR scores for animals that could perform at least one plantar step, non-stepping animals, and sham rats revealed a strong relationship between the ability to generate plantar steps and the IFFT and PWR scores (Fig. 2B).

Long-term step training alone fails to improve motor function

To determine if training alone could promote improvements in hindlimb motor function after transection we compared the performance of media-untrained and media-trained rats using the three analyses of motor performance described earlier. At 4 months post-surgery, 0/9 media-untrained rats performed plantar steps and at 7 months only 1/9 rats could complete a single plantar step (Fig. 1F). In comparison, 4/9 media-trained rats performed at least one plantar step at 4 months and 3/9 could execute 1–2

steps at 7 months (Fig. 1G). Only 1/9 media-untrained and 1/9 media-trained rats improved their stepping performance between 4 and 7 months post-transection ($P = 0.21$). Media-trained rats generated more plantar steps than media-untrained animals at 4 months post-surgery (3 ± 3 versus 0 ± 0; mean $\pm$ SEM; $P = 0.01$), but not at 7 months (1 ± 1 versus 0 ± 0; $P = 0.08$). Overall, rats in both media-treated groups dragged the dorsal surface of their feet and rarely initiated plantar-placed steps while unassisted on the treadmill (Supplementary Videos 1 and 2).

When we compared the movement frequency of the internal joint angles described with the IFFT there were no significant differences at 7 months between media-untrained and media-trained rats (Fig. 2C; $P = 0.49$). Likewise, the hindlimb movement based on normalized PWR values did not differ between media-untrained and media-trained rats at 7 months post-transection (Fig. 2C; $P = 0.36$). Therefore, 6 months of treadmill step-training alone failed to produce an improvement in the quantitative measures of hindlimb stepping analysed.

OEG transplantation alone improves plantar stepping

We compared OEG-untrained and media-untrained rats to assess the effectiveness of OEG transplantation alone in restoring hindlimb motor function. In contrast to the lack of stepping found in media-untrained rats, 3/10 OEG-untrained rats performed 1–15 plantar steps at 4 months, and 5/10 rats completed 1–26 steps at 7 months post-transection (Fig. 1F and H). The average number of plantar steps performed was significantly higher in the OEG-untrained rats compared to the media-untrained rats at both 4 (2 ± 2 versus 0 ± 0; $P = 0.04$) and 7 (6 ± 3 versus 0 ± 0; $P = 0.02$) months post-transection. However, the improvement in the number of plantar steps executed at 7 versus 4 months did not differ significantly between the OEG-untrained (4 ± 2) and media-untrained groups (0 ± 0; $P = 0.16$).

Additional kinematic evaluations at 7 months found that OEG-untrained and media-untrained animals did not differ significantly in the IFFT measure of stepping frequency (Fig. 2C; $P = 0.09$) or in the mean stepping quality analysed by PWR scores (Fig. 2C; $P = 0.18$) despite exceptional stepping performances by individual animals (Supplementary Video 3). The significant differences observed in the ability to perform plantar-placed steps, but not in the measures of step frequency or mean stepping quality, reflect the different aspects of locomotion captured by each measure. The number of plantar-placed steps is a discontinuous measure that indicates a high degree of capability, but cannot discriminate intermediate levels of recovery. The hindlimbs can regain the ability to oscillate with a reasonably consistent rhythm and even display some interjoint coordination at an intermediate level of recovery. The ability for some animals to obtain low and intermediate levels of recovery are reflected in the IFFT and PWR scores even when the animals are incapable of plantar stepping. However, greater IFFT and PWR scores in groups that exhibited many plantar steps shows that plantar stepping was due to increasingly normal stepping kinematics.

OEG transplantation combined with step-training facilitates long-term recovery of hindlimb stepping

To evaluate the effect of OEG transplantation and step training we compared plantar stepping in the OEG-trained group to the three other groups. At the end of the study, 8/10 OEG-trained rats could plantar step (Supplemental Video 4) in contrast to 1/9 media-untrained, 3/9 media-trained and 5/10 OEG-untrained rats. OEG-trained rats generated significantly more plantar steps than media-untrained rats at both 4 (4 ± 2 versus 0 ± 0; $P = 0.02$) and 7 (9 ± 2 versus 0 ± 0; $P < 0.001$) months, and more steps than media-trained rats at 7 months (9 ± 2 versus 1 ± 1; $P = 0.005$, Fig. 1F, G and I). However, the mean number of steps completed by the OEG-trained rats did not differ from the OEG-untrained rats at 4 (4 ± 2 versus 2 ± 2; $P = 0.27$) or 7 (9 ± 2 versus 6 ± 3; $P = 0.07$) months after transection (Fig. 1H and I).

We next compared the mean improvement in stepping abilities from 4 to 7 months by group. Only the OEG-trained group performed significantly more plantar steps at 7 than at 4 months (9 ± 2 versus 4 ± 2; $P = 0.03$; Fig. 1I). To further investigate the changes in stepping ability over time, we compared the improvement for each animal by calculating the difference in plantar steps between 4 and 7 months. A total of 8/10 OEG-trained rats improved their plantar-stepping performances between 4 and 7 months compared to 1/9 in each of the media-treated groups and 3/10 in the OEG-untrained group. In addition, the OEG-trained group showed a significantly greater improvement in the number of plantar steps (improvement of 5 ± 1 steps) than any of the other groups (media-untrained, 0 ± 0, $P \leqslant 0.001$; media-trained, -3 ± 3, $P \leqslant 0.001$; OEG-untrained, 4 ± 2, $P = 0.03$). Thus the 4 to 7-month improvement in plantar stepping for the OEG-trained group was unique, when considered as group means or paired comparisons of individual improvement, since no other comparisons between groups differed significantly.

Comparisons of step frequency and step shape were significantly different between OEG-trained and media-untrained animals at 7 months (Fig. 2C; IFFT; $P = 0.03$; PWR; $P = 0.03$), while OEG-untrained and media-untrained animals did not differ. OEG-trained and media-trained rats differed in IFFT scores (Fig. 2C; $P = 0.03$), but not in PWR scores (Fig. 2C; $P = 0.10$), whereas OEG-untrained animals did not differ from media-trained animals in

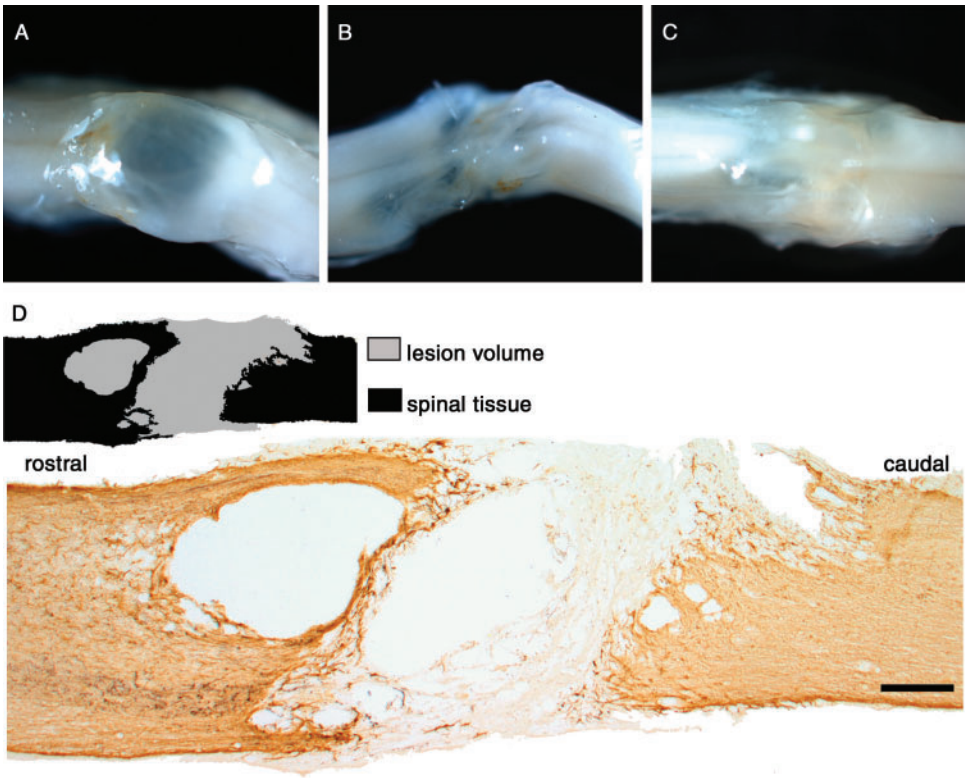

Fig. 3 Tissue sparing at the transection site occurs with OEG transplantation. (**A–C**) Transected spinal cords demonstrate a range of cavitation. A spinal cord from a media-untrained (A) rat shows large transparent cavities and little evidence of regeneration, while much less cavitation is apparent in the injury site from a second media-untrained (B) rat. An OEG-trained animal demonstrates an opaque injury site devoid of pronounced cavitations (C). (**D**) A 25-μm-thick sagittal spinal cord section immunostained for GFAP (amber-brown) containing the transection site and a drawing illustrating the identification of the GFAP-positive tissue (black area in drawing) and the GFAP-negative transection site (gray) separating the rostral and caudal stumps. Scale bar: D, 400 μm.

either measure. Although the kinematics of the OEG-untrained and OEG-trained groups did not differ in either the IFFT (Fig. 2C; $P = 0.24$) or PWR ($P = 0.15$) scores, the two groups did show differences when compared to media-injected and sham rats. The kinematics of OEG-trained rats were not significantly different from those of sham rats (Fig. 2C; IFFT; $P = 0.48$; PWR; $P = 0.11$), but shams had significantly different PWR scores from OEG-untrained animals ($P = 0.01$). Sham rats also had significantly different stepping kinematics as measured by IFFT and PWR scores compared to both media groups ($P < 0.02$ for all comparisons).

When the size and directionality of the means and the mean differences of all four groups are taken together, we found that: (1) training alone did not significantly improve motor recovery, (2) OEG transplantation alone resulted in a significant increase in the number of plantar steps performed and (3) the combination of training and OEG transplantation produced the highest percentage of spinal rats that plantar stepped, differences in kinematics when compared to media groups, and significant improvements in their stepping abilities between the 4 and 7-month time points. Videos of the best hindlimb stepping from each of the four groups are available in the Supplemental movies 1–4.

OEG transplantation promotes tissue sparing

Spinal cord tissue around the transection site typically degenerates and forms cavitations and scar tissue that inhibit axonal regeneration (Li *et al*., 1997, 2003; Ramón-Cueto *et al*., 1998). While we confirmed a complete transection of the spinal cord by demonstrating a GFAP-negative scar separating the rostral and caudal spinal cord stumps in all transected animals (Supplemental Figs. 2 and 3), we detected considerable variation in the morphology of the lesion sites among our experimental groups. Some rats displayed large transparent cavities that separated their two opaque spinal cord stumps (Fig. 3A), while others contained smaller cavities (Fig. 3B) or appeared almost solid (Fig. 3C). Generally, OEG-injected rats that could generate plantar steps had more solid appearing injury sites than OEG-injected non-steppers or any of the media-injected animals.

To quantify the tissue-repair effect, we measured the cavities and the GFAP-negative areas to determine the total

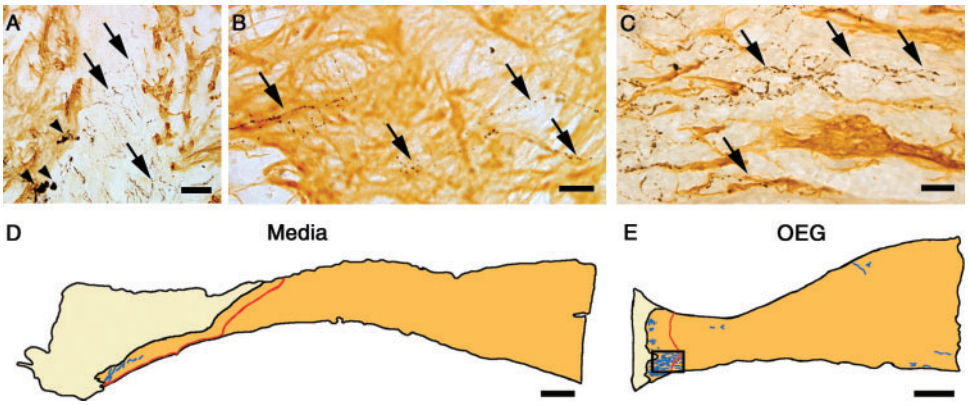

Fig. 4 Noradrenergic axons extend into and through the GFAP-negative scar. Sagittal sections are oriented with rostral to the left and dorsal to the top in this and the following figure. (**A**) DBH-positive axons (arrows) extend between the rostral GFAP-positive spinal cord and the GFAP-negative scar in a media-injected rat. Large bulbous endings (arrowheads) are present at the rostral border of the injury site. (**B**) In an OEG-injected animal, DBH-positive fibres (arrows) extend into the GFAP-positive caudal spinal cord. (**C**) DBH-positive fibres (arrows) course into the GFAP-positive caudal stump in an OEG-injected animal, with some fibres extending more than 250 μm from the caudal border of the GFAP-negative zone (C is enlarged from the boxed area in E) (**D**, **E**) Merged camera lucida drawings of seven 25-μm-thick sagittal sections from the caudal stump of a media-untrained (D) and an OEG-untrained (E) rat. The GFAP-negative scar tissue is light beige, the caudal GFAP-positive spinal cord is dark beige, and the red line demarcates a distance of 250 μm caudal to the GFAP-negative border. Many more DBH-positive fibres are detected caudal to the 250 μm boundary in OEG- compared to media-injected animals. Scale bars: A, 50 μm; B, C, 25 μm; D, E, 400 μm.

volume that separates the GFAP-positive rostral and caudal stumps. We analysed 10–15% of the sagittal sections throughout the lesion block and subtracted the GFAP-positive zones from the total area of the block to estimate the volume of the injury site (Fig. 3D, gray area of drawing). The average lesion volumes of OEG-injected rats were significantly smaller than those of media-injected animals ($1.8 \pm 0.33\,mm^3$ versus $3.3 \pm 0.52\,mm^3$; $P = 0.02$), suggesting that OEG cells induce a process conducive to tissue repair.

OEG transplantation promotes noradrenergic fibre regeneration

Descending noradrenergic projections and their pharmacological agonists can modulate and induce stepping in spinal transected rats, and previous reports show that regeneration of these fibres correlates with improvements in motor function (Ramón-Cueto *et al.*, 1998, 2000; López-Vales *et al.*, 2006). Therefore, we analysed the descending DBH-positive axons around the injury site and in the adjacent caudal stump. In both media- and OEG-injected animals DBH-positive axons occupy the rostral spinal cord, readily extend into the GFAP-negative scar (arrows, Fig. 4A), and enter the GFAP-positive caudal spinal cord (arrows, Fig. 4B and C). However, significantly more DBH-positive fibres per mm^3 of tissue reside in the GFAP-positive caudal stump in OEG- than in media-injected rats (Table 1, 14 ± 7 versus 1 ± 0; $P = 0.03$).

Additionally, DBH-positive fibre distribution patterns differ between media- and OEG-injected animals (Fig. 4D and E). In media-injected animals only 7% of the DBH-positive axons extend further than 250 μm beyond the lesion site compared to 40% in OEG-injected animals, results suggesting that these fibres sprout short distances in transected animals. Furthermore, while the difference in DBH-positive fibre density between the media- and OEG-injected animals within 250 μm of the caudal GFAP-negative zone was not statistically significant (10 ± 3 versus 65 ± 37; $P = 0.09$), the fibre density in the remainder of the block caudal to the lesion (>250 μm) was greater in OEG-injected animals (0 ± 0 versus 7 ± 3; $P = 0.01$). Thus, OEG transplantation is sufficient to promote substantial regeneration of DBH-positive fibres and this regeneration could contribute to recovery of motor function after a complete transection.

Prevalence of serotonergic fibres in the caudal stump following transection

Previous studies suggest that the presence of 5-HT-positive fibres caudal to a complete spinal transection provides evidence of regeneration that is associated with improved motor function (Cheng and Olson, 1995; Ramón-Cueto *et al.*, 2000; Lu *et al.*, 2001, 2002; López-Vales *et al.*, 2006). We found numerous 5-HT-positive axons immediately rostral to the transection site in all spinal rats examined, but few immunoreactive axons entered the GFAP-negative scar (Fig. 5A). Additionally, we found several 5-HT-positive neurons immediately rostral to the transection site (arrow, Fig. 5B). Only in OEG-injected animals did we observe 5-HT-positive axons coursing between the glial scar and the GFAP-positive caudal stump (Fig. 5C and D) and axons spanning the GFAP-negative zone (Fig. 5E–G). Since media-injected rats did not contain serotonergic fibres spanning the lesion site, the 5-HT-positive axons crossing the transection in OEG-treated rats most likely represent regeneration.

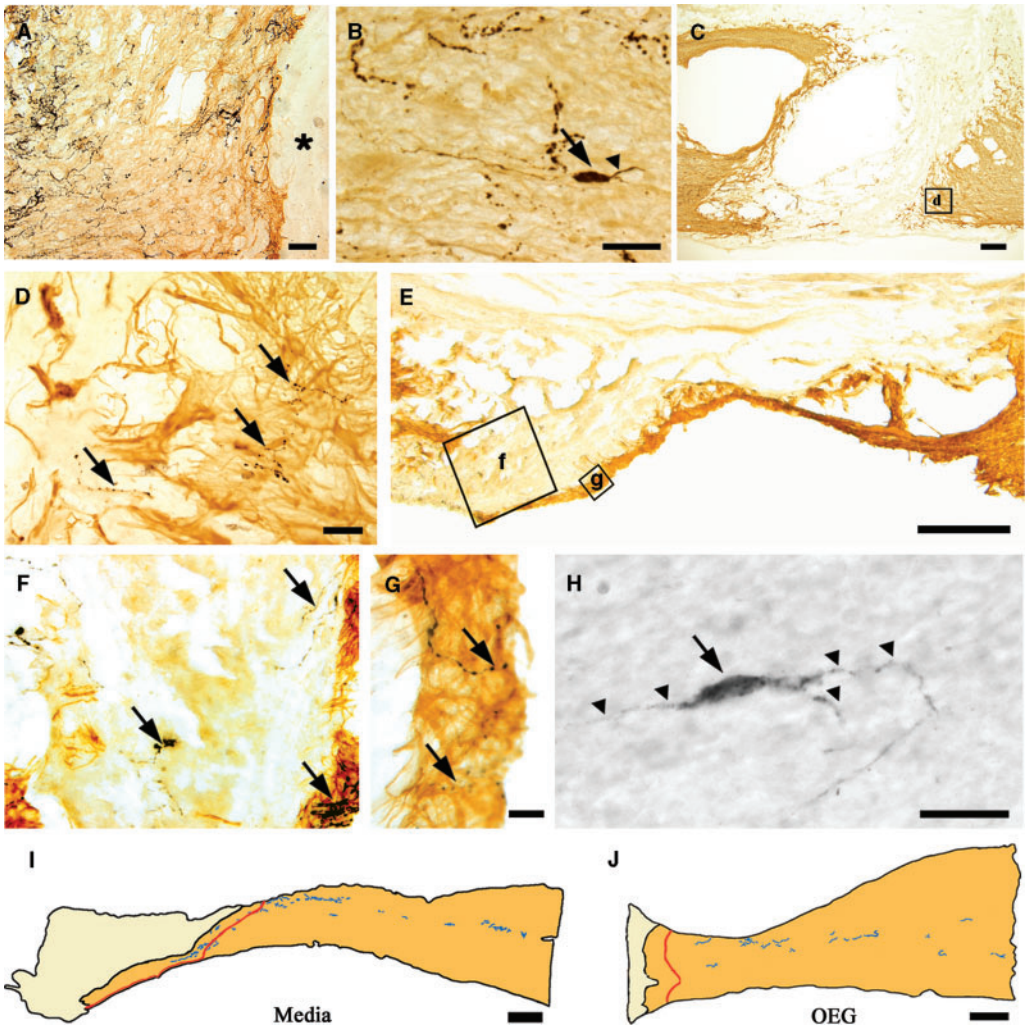

Fig. 5 Serotonergic axons and cell bodies are found rostral (to the left) and caudal to the injury site. (**A**) 5-HT-positive (black) axons in the rostral GFAP-positive (amber-brown) spinal cord of a media-untrained animal terminate at the GFAP-negative scar (asterisk). (**B**) A 5-HT-positive interneuron (arrow) just rostral to the GFAP-negative zone of a media-injected rat. A branched process (arrowhead) extends from this bipolar neuron. (**C**, **D**) The injury site of an OEG-trained rat double-labelled for GFAP and 5-HT immunoreactivity. 5-HT-positive axons (D, arrows) course into the caudal GFAP-positive spinal cord (boxed area in C is enlarged in D). (**E**–**G**) 5-HT-positive fibres span the GFAP-negative scar of an OEG-untrained animal (boxed areas in E are enlarged in F and G). 5-HT-positive fibres (arrows) extend through the scar and enter the GFAP-positive caudal spinal cord. (**H**) Several processes (arrowheads) extend from this 5-HT-positive interneuron (arrow) found caudal to the transection site (~T10) in a media-injected rat. (**I**, **J**) Merged camera lucida drawings of seven 25-μm-thick sagittal sections of caudal spinal cord stumps from a media-untrained (I) and an OEG-untrained (J) animal demonstrate similar patterns of 5-HT-labelled axons. The GFAP-negative scar tissue is light beige, the caudal GFAP-positive spinal cord is dark beige, and the red line demarcates a distance of 250 μm from the GFAP-negative zone. Scale bars A, 50 μm; C, 200 μm; B, D, F, G, H, 25 μm; E, 100 μm; I, J, 400 μm.

More detailed analyses within the area directly caudal to the lesion unexpectedly showed numerous 5-HT-positive fibres in both media- and OEG-injected groups (Table 1). Previous studies reported the presence of both 5-HT-positive interneurons (Newton *et al.*, 1986; Newton and Hamill, 1988) and fibres (Guest *et al.*, 1997; Fouad *et al.*, 2005) in the caudal stump following spinal transection. Similarly, we detected serotonergic fibres and occasional cell bodies in the caudal stump of media-injected animals (Fig. 5H). Furthermore, the 5-HT-positive fibre distribution was similar in the media- and OEG-injected groups, with 88 and 92% of the 5-HT-positive fibres, respectively, located more than 250 μm past the injury site (Fig. 5I and J). There was no difference in the 5-HT-positive fibre density below the transection site in media- and OEG-injected rats (18 ± 3 versus 15 ± 6; $P = 0.70$).

Discussion

Following a complete spinal cord transection in adult rats, we show that olfactory bulb-derived OEG transplantation significantly improves plantar stepping on a treadmill for at least 7 months, the end point of this study. Furthermore,

the combination of OEG transplantation and extensive step training resulted in the highest number of paraplegic rats that could step and the largest percentage of rats that improved their stepping abilities between 4 and 7 months post-injury. This improvement in hindlimb plantar stepping over time was the most notable feature of the OEG-trained group and rarely observed in media-injected rats. We also identified significantly more noradrenergic axons in the caudal stumps of OEG- than media-injected animals, findings that suggest regeneration of the coeruleospinal pathway across the transection site. Surprisingly, we found similar numbers of serotonergic axons in the caudal stump of both media- and OEG-injected rats and identified serotonergic interneurons as a likely source.

Combining OEG transplantation with step-training facilitates plantar stepping and hindlimb kinematics

While previous studies report that OEG transplantation restores partial hindlimb motor function in adult paraplegic rats (Ramón-Cueto *et al*., 2000; Lu *et al*., 2001, 2002; López-Vales *et al*., 2006), few objective measurements of the improved function were used. We used three quantitative measures to assess locomotor performance, with each measure reflecting a different feature of locomotion. The percentage of paraplegic rats that could plantar step 7 months after transection varied dramatically among our treatment groups. A total of only 11% of media-untrained or 33% of media-trained rats performed 1 or 2 plantar steps, while 50% of the OEG-untrained and 80% of OEG-trained rats generated 1 to 26 plantar steps. Thus OEG transplantation improved hindlimb stepping on a treadmill and long-term training appeared to enhance and maintain the OEG effect on stepping recovery over the 7-month period studied.

A novel finding in the present study is that the effects of a transplantation intervention can be enhanced when combined with training for a specific motor task. Ramón-Cueto *et al*. (2000) also reported improved performance in a climbing test 3 to 7 months after a complete transection and OEG implantation in adult rats. Although technically these rats were not trained, they did practice the climbing task repetitively. Using the same OEG transplantation techniques and training period but a different motor task, i.e. treadmill step training, our results are consistent with the presence of activity-dependent mechanisms that can augment the effects of OEG transplantation. Additionally, only the OEG-trained rats continued to improve throughout the 7-month study. This improvement in motor performance during the period between 4 and 7 months post-transection is particularly promising because in almost all chronic models of spinal cord injury there tends to be a decline, or at best a plateau, in locomotor function several months after a complete spinal transection in rats (de Leon and Acosta, 2006) and cats (Lovely *et al*., 1986), even if step training is continued. In the present study only 11% of the media-treated rats compared with 55% of the OEG-treated rats improved in their ability to plantar step between 4 and 7 months, arguably the most difficult locomotor function to achieve post-transection.

Given the number of studies that report successful recovery of weight-bearing stepping in cats following a complete spinal cord transection (Edgerton *et al*., 2004), it was a surprising but consistent finding that the media-injected, trained rodents in the present study did not display a training effect. Cats can regain the ability to generate full weight-bearing steps with only step training and even some untrained cats can reach some level of recovery spontaneously. Rats transected as neonates also can be trained to generate weight-bearing steps (Kubasak *et al*., 2005). In contrast, adult rats and mice can achieve this level of stepping performance after a complete spinal cord transection only when step training is combined with other treatments, such as the administration of pharmacological agents (Fong *et al*., 2005), epidural stimulation (Ichiyama *et al*., 2005), or transplantation of embryonic raphe cells (Ribotta *et al*., 2000). The explanation for this fundamental difference between cats and rodents remains unclear.

While the mechanisms by which training could improve the performance in spinalized OEG-injected rats are not known, training could facilitate axon regeneration, and/or provide protection for the neurons and glia near the injury site. OEG secrete the neurotrophic factors NGF and BDNF (Ramón-Cueto and Avila, 1998; Chuah and West, 2002), promote neurite sprouting both by cell contact and through the secretion of soluble factors *in vitro* (Chuah *et al*., 2004), as well as facilitate cell survival. Exercise increases neurotrophic factors (BDNF, NT-3) and their Trk receptors in the spinal cord (Gómez-Pinilla *et al*., 2001, 2002) and BDNF facilitates intrinsic spinal cord reorganization (Ying *et al*., 2005). Thus our OEG-injected groups with or without exercise may have benefited from neurotrophic factors that enhance axonal regeneration across the transection. An important and fundamental concept revealed by the present results is that the final outcome of combining training with cell transplantation interventions, such as OEG, can be influenced by activity of the neural circuits that generate the practiced motor tasks.

OEG transplantation promotes tissue sparing and axonal regeneration

OEG transplantation has a protective effect on the injured spinal cord by limiting cavitations and scar formation following a complete transection (Ramón-Cueto *et al*., 2000; López-Vales *et al*., 2006) and our results are consistent with these reports. Therefore, by limiting tissue degeneration, axonal dieback, and the formation of cavitations following injury, regenerating axons in OEG-transplanted animals

may have a smaller and possibly more permissive milieu to traverse.

Similar to previous reports (Ramón-Cueto *et al*., 2000; López-Vales *et al*., 2006), we detected DBH-positive axons extending relatively long distances into the GFAP-positive caudal spinal cords in OEG-treated rats. While some immunoreactive fibres in media-injected rats coursed relatively short (<250 μm) distances between the GFAP-negative scar and the GFAP-positive caudal stump, the density of DBH-positive fibres throughout the remainder of the transection block (i.e. >250 μm) was significantly lower in media- than OEG-injected animals. These data suggest that regeneration of DBH-positive axons following a complete spinal cord transection could contribute to the improved motor function. Further experiments will be essential to confirm this observation.

As a second indicator of axonal regeneration we analysed the presence of 5-HT-positive fibres in the caudal stump. While we found evidence of serotonergic fibres regenerating across the transection site only in OEG-injected animals as reported previously (Ramón-Cueto *et al*., 2000; Lu *et al*., 2001, 2002; López-Vales *et al*., 2006), we also found immunoreactive fibres and a few 5-HT-positive interneurons caudal to the GFAP-negative zone in both media- and OEG-treated rats. Based on these findings and previous reports of 5-HT-positive interneurons (Newton *et al*., 1986; Newton and Hamill, 1988) and fibres (Guest *et al*., 1997; Fouad *et al*., 2005) caudal to the transection site, the 5-HT-labelled fibres detected in media-injected rats are likely derived from an endogenous source such as the serotonergic interneurons. After a complete spinal-cord transection, biochemical studies detect 5-HT levels that are 2–15% of normal (references reviewed by Newton and Hamill, 1988 and Schmidt and Jordan, 2000) and a recent study suggests that these low levels of residual 5-HT might function to produce calcium currents that contribute to muscle spasticity (Li *et al*., 2007). In the present study the density of 5-HT-immunopositive axons below the spinal cord transection was variable and independent of the training or OEG transplantation status. Thus, we conclude that the localization of 5-HT-immunoreactive axons caudal to the transection site, i.e. in those regions where serotonergic interneurons are located, is not a reliable indicator of axonal regeneration following spinal cord transection.

Conclusions

These results confirm previous observations that olfactory bulb-derived OEG implantation can improve motor function in complete spinal rats. Additionally, only rats that received step training in combination with OEG transplantation improved their stepping ability from 4 to 7 months post-transection. Therefore, our results support the concept that task-specific training can further improve the functional potential of a neuronal regenerative therapy. These observations have important implications when designing repair strategies for spinal cord injury in humans.

Supplementary materials

Supplementary materials are available at *Brain* online.

Acknowledgements

We thank Drs A. Garfinkel and J. Gornbein as well as D. Tran for expert assistance in the statistical analyses of the stepping data; S. Zdunowski for help with engineering and robotics; M. Herrera and Dr G. Lawson for their aid with postsurgical animal care; Dr F. Santos-Benito for instruction and assistance in OEG cultures; A. Babayan for figure revisions; and numerous UCLA undergraduates for assistance with animal care, animal training and data analysis. Funding from the Christopher Reeve Paralysis Foundation (PA-1-0102-2, PAC1-0102-2, PEP) and NINDS (R21NS42000-01, PEP; RO1NS54159, VRE).

References

Barbeau H, Chau C, Rossignol S. Noradrenergic agonists and locomotor training affect locomotor recovery after cord transection in adult cats. Brain Res Bull 1993; 30: 387–93.

Barbeau H, Rossignol S. Enhancement of locomotor recovery following spinal cord injury. Curr Opin Neurol 1994; 7: 517–24.

Barbeau H, Rossignol S. Recovery of locomotion after chronic spinalization in the adult cat. Brain Res 1987; 412: 84–95.

Barbeau H, Rossignol S. Initiation and modulation of the locomotor pattern in the adult chronic spinal cat by noradrenergic, serotonergic and dopaminergic drugs. Brain Res 1991; 546: 250–60.

Basso DM, Beattie MS, Bresnahan JC. A sensitive and reliable locomotor rating scale for open field testing in rats. J Neurotrauma 1995; 12: 1–21.

Burke-Hubbard B. The world according to wavelets. Wellesley, MA: AK Peters; 1998.

Chandler CE, Parsons LM, Hosang M, Shooter EM. A monoclonal antibody modulates the interaction of nerve growth factor with PC12 cells. J Biol Chem 1984; 259: 6882–9.

Cheng H, Olson L. A new surgical technique that allows proximodistal regeneration of 5-HT fibers after complete transection of the rat spinal cord. Exp Neurol 1995; 136: 149–61.

Chuah MI, Choi-Lundberg D, Weston S, Vincent AJ, Chung RS, Vickers JC, et al. Olfactory ensheathing cells promote collateral axonal branching in the injured adult rat spinal cord. Exp Neurol 2004; 185: 15–25.

Chuah MI, West AK. Cellular and molecular biology of ensheathing cells. Microsc Res Tech 2002; 58: 216–27.

Côte M-P, Gossard J-P. Step training-dependent plasticity in spinal cutaneous pathways. J Neurosci 2004; 24: 11317–27.

Côte M-P, Menard A, Gossard J-P. Spinal cats on the treadmill: changes in load pathways. J Neurosci 2003; 23: 2789–96.

de Leon RD, Acosta CA. Effect of robotic-assisted treadmill training and chronic quipazine treatment on hindlimb stepping in spinally transected rats. J Neurotrauma 2006; 23: 1147–63.

de Leon RD, Hodgson JA, Roy RR, Edgerton VR. Locomotor capacity attributable to step training versus spontaneous recovery after spinalization in adult cats. J Neurophysiol 1998; 79: 1329–40.

de Leon RD, Tamaki H, Hodgson JA, Roy RR, Edgerton VR. Hindlimb locomotor and postural training modulates glycinergic inhibition in the spinal cord of the adult spinal cat. J Neurophysiol 1999; 82: 359–69.

Dietz V. Spinal cord pattern generators for locomotion. Clin Neurophysiol 2003; 114: 1379–89.

Edgerton VR, Tillakaratne NJK, Bigbee AJ, de Leon RD, Roy RR. Plasticity of the spinal neural circuitry after injury. Annu Rev Neurosci 2004; 27: 145–67.

Efron B, Tibshirani R. Statistical data analysis in the computer age. Science 1991; 253: 390–5.

Fong AJ, Cai LL, Otoshi CK, Reinkensmeyer DJ, Burdick JW, Roy RR, et al. Spinal cord-transected mice learn to step in response to quipazine treatment and robotic training. J Neurosci 2005; 25: 11738–47.

Fouad K, Schnell L, Bunge MB, Schwab ME, Liebscher T, Pearse DD. Combining Schwann cell bridges and olfactory-ensheathing glia grafts with chondroitinase promotes locomotor recovery after complete transection of the spinal cord. J Neurosci 2005; 25: 1169–78.

García-Alías G, López-Vales R, Forés J, Navarro X, Verdú E. Acute transplantation of olfactory ensheathing cells or Schwann cells promotes recovery after spinal cord injury in the rat. J Neurosci Res 2004; 75: 632–41.

Giroux N, Reader TA, Rossignol S. Comparison of the effect of intrathecal administration of clonidine and yohimbine on the locomotion of intact and spinal cats. J Neurophysiol 2001; 85: 2516–36.

Gómez-Pinilla F, Ying Z, Opazo P, Roy RR, Edgerton VR. Differential regulation by exercise of BDNF and NT-3 in rat spinal cord and skeletal muscle. Eur J Neurosci 2001; 13: 1078–84.

Gómez-Pinilla F, Ying Z, Roy RR, Molteni R, Edgerton VR. Voluntary exercise induces a BDNF-mediated mechanism that promotes neuroplasticity. J Neurophysiol 2002; 88: 2187–95.

Grillner S, Markram H, De Schutter E, Silberberg G, LeBeau FEN. Microcircuits in action–from CPGs to neocortex. Trends Neurosci 2005; 28: 525–33.

Guertin PA. Role of NMDA receptor activation in serotonin agonist-induced air-stepping in paraplegic mice. Spinal Cord 2004; 42: 185–90.

Guest JD, Rao A, Olson L, Bunge MB, Bunge RP. The ability of human Schwann cell grafts to promote regeneration in the transected nude rat spinal cord. Exp Neurol 1997; 148: 502–22.

Harkema SJ, Hurley SL, Patel UK, Requejo PS, Dobkin BH, Edgerton VR. Human lumbosacral spinal cord interprets loading during stepping. J Neurophysiol 1997; 77: 797–811.

Iannotti C, Zhang YP, Shields LBE, Han Y, Burke DA, Xu X-M, et al. Dural repair reduces connective tissue scar invasion and cystic cavity formation after acute spinal cord laceration injury in adult rats. J Neurotrauma 2006; 23: 853–65.

Ichiyama RM, Gerasimenko YP, Zhong H, Roy RR, Edgerton VR. Hindlimb stepping movements in complete adult spinal rats induced by epidural spinal cord stimulation. Neurosci Letters 2005; 383: 339–44.

Imaizumi T, Lankford KL, Waxman SG, Greer CA, Kocsis JD. Transplanted olfactory ensheathing cell remyelinate and enhance axonal conduction in the demyelinated dorsal columns of the rat spinal cord. J Neurosci 1998; 18: 6176–85.

Kato T, Honmou O, Uede T, Hashi K, Kocsis JD. Transplantation of human olfactory ensheathing cells elicits remyelination of demyelinated rat spinal cord. Glia 2000; 30: 209–18.

Kubasak MD, Hedlund E, Roy RR, Carpenter EM, Edgerton VR, Phelps PE. L1 CAM expression is increased surrounding the lesion site in rats with complete spinal cord transection as neonates. Exp Neurol 2005; 194: 363–75.

Li X, Murray K, Harvey PJ, Ballou EW, Bennett DJ. Serotonin facilitates a persistent calcium current in motoneurons of rats with and without chronic spinal cord injury. J Neurophysiol 2007; 97: 1236–46.

Li Y, Field PM, Raisman G. Repair of adult rat corticospinal tract by transplants of olfactory ensheathing cells. Science 1997; 277: 2000–2.

Li Y, Decherchi P, Raisman G. Transplantation of olfactory ensheathing cells into spinal cord lesions restores breathing and climbing. J Neurosci 2003; 23: 727–31.

López-Vales R, Forés J, Verdú E, Navarro X. Acute and delayed transplantation of olfactory ensheathing cells promote partial recovery after complete transection of the spinal cord. Neurobiol Dis 2006; 21: 57–68.

Lovely RG, Gregor RJ, Roy RR, Edgerton VR. Effects of training on the recovery of full-weight-bearing stepping in the adult spinal cat. Exp Neurol 1986; 92: 421–35.

Lu J, Féron F, Ho SM, Mackay-Sim A, Waite PME. Transplantation of nasal olfactory tissue promotes partial recovery in paraplegic rats. Brain Res 2001; 889: 344–57.

Lu J, Féron F, Mackay-Sim A, Waite PME. Olfactory ensheathing cells promote locomotor recovery after delayed transplantation into transected spinal cord. Brain 2002; 125: 14–21.

Newton BW, Hamill RW. The morphology and distribution of rat serotoninergic intraspinal neurons: an immunohistochemical study. Brain Res Bull 1988; 20: 349–60.

Newton BW, Maley BE, Hamill RW. Immunohistochemical demonstration of serotonin neurons in autonomic regions of the rat spinal cord. Brain Res 1986; 376: 155–63.

Petruska JC, Ichiyama RM, Jindrich DL, Crown ED, Tansey KE, Roy RR, et al. Changes in motoneuron properties and synaptic inputs related to step training after spinal cord transection in rats. J Neurosci 2007; 27: 4460–71.

Ramón-Cueto A, Avila J. Olfactory ensheathing glia: properties and function. Brain Res Bull 1998; 46: 175–87.

Ramón-Cueto A, Nieto-Sampedro M. Regeneration into the spinal cord of transected dorsal root axons is promoted by ensheathing glia transplants. Exp Neurol 1994; 127: 232–44.

Ramón-Cueto A, Plant GW, Avila J, Bunge MB. Long-distance axonal regeneration in the transected adult rat spinal cord is promoted by olfactory ensheathing glia transplants. J Neurosci 1998; 18: 3803–15.

Ramón-Cueto A, Cordero MI, Santos-Benito FF, Avila J. Functional recovery of paraplegic rats and motor axon regeneration in their spinal cords by olfactory ensheathing glia. Neuron 2000; 25: 425–35.

Ribotta MG, Provencher J, Feraboli-Lohnherr D, Rossignol S, Privat A, Orsal D. Activation of locomotion in adult chronic spinal rats is achieved by transplantation of embryonic raphe cells reinnervating a precise lumbar level. J Neurosci 2000; 20: 5144–52.

Roy RR, Hodgson JA, Lauretz SD, Pierotti DJ, Gayek RJ, Edgerton VR. Chronic spinal cord-injured cats: surgical procedures and management. Lab Anim Sci 1992; 42: 335–43.

Schmidt BJ, Jordan LM. The role of serotonin in reflex modulation and locomotor rhythm production in the mammalian spinal cord. Brain Res Bull 2000; 53: 689–710.

Tillakaratne NJK, de Leon RD, Hoang TX, Roy RR, Edgerton VR, Tobin AJ. Use-dependent modulation of inhibitory capacity in the feline lumbar spinal cord. J Neurosci 2002; 22: 3130–43.

Veasey SC, Fornal CA, Metzler CW, Jacobs BL. Response of serotonergic caudal raphe neurons in relation to specific motor activities in freely moving cats. J Neurosci 1995; 15: 5346–59.

Wernig A, Nanassy A, Muller S. Laufband (LB) therapy in spinal cord lesioned persons. Prog Brain Res 2000; 128: 89–97.

Xu XM, Zhang S-X, Li H, Aebischer P, Bunge MB. Regrowth of axons into the distal spinal cord through a Schwann-cell-seeded mini-channel implanted into hemisected adult rat spinal cord. Eur J Neurosci 1999; 11: 1723–40.

Ying Z, Roy RR, Edgerton VR, Gómez-Pinilla F. Exercise restores levels of neurotrophins and synaptic plasticity following spinal cord injury. Exp Neurol 2005; 193: 411–19.