

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

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For Peer Review

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

Running title: Locomotion in OEG-treated spinal transected rats

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Abstract

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 were the only group to significantly improve their 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-labeled 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.

Key words: 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 = 0.1M Tris buffer with 1.4% NaCl and 0.1% bovine serum albumin

OEG transplantation reportedly facilitates axon regeneration, reduces tissue loss, and improves motor performance in different models of spinal cord injury (Ramón-Cueto et al., 1994; Li et al., 1997, 2003; Imaizumi et al., 1998; Kato et al., 2000; García-Álías 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 et al., 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 behavior 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 fibers 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 two 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 anesthetized 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 μ m 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 two days of recovery. Bladder expressions were performed three times a day for three days and then twice daily, at 12 hour 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 five minutes of step training and the training time was increased by five minutes each week for the first month. For the next five months, rats were trained 20 minutes/day, five

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 hours of manual step training.

Stepping ability was evaluated at 4 and 7 months post-surgery at a treadmill speed of 13 cm/sec 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 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 anesthetized with Ketamine (0.9 μ l/gram) and Anased (0.5 μ l/gram)

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 0.1M Tris buffer containing 1.4% NaCl and 0.1% bovine albumin (TBS), followed by a 30-minute presoak in 0.3% H_2O_2 and 0.1% sodium azide. After a 0.8% Triton presoak for 15 minutes, sections were incubated in 1.5% normal horse serum with 0.1% Triton and an additional blocking step to reduce nonspecific 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 one hour 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 fibers, 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-labeling 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, 3).

Morphological analyses

Animals selected for statistical analyses represented a range of stepping abilities (see 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-labeled immunoreactivity. We counted the number of labeled axons caudal to the transection site from 3 rats per antibody in each experimental group. For each animal, we analyzed between 18 and 50 sections/antibody to quantify the number of labeled axons caudal to the transection site. We used Openlab software (Improvision Inc., Lexington,

MA) to identify fibers 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 below) to calculate the areal density. We grouped fibers into those that extend up to 250 μm beyond the caudal GFAP-interface and those coursing further distances into the caudal stump (or endogenous fibers) (Xu et al., 1999).

To analyze tissue sparing we examined 10-15% of the sections distributed evenly throughout the transection site from the same 3 animals per experimental group reported above. Starting with the first identifiable section, sequential sections at 175-200 μm intervals were analyzed 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 7 alternating 25 μm -thick sagittal sections were scanned, overlaid, and merged. Fiber 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 seconds of continuous stepping using the FFT, and the frequency at peak power was identified. The mean and standard deviation 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 centered at the mean frequency at peak power with a width of two standard deviations. 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/sec. 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 to 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 behavior 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 pre-determined 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 analyze 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 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, B), during initial paw contact (Fig. 1C), and remains on the treadmill belt throughout the stance phase (Fig. 1D, E). Step failure occurs when the animal drags its hindlimbs on the treadmill. We analyzed 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 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).

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 peak wavelet resemblance (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 above. 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 vs. 0 ± 0 ; mean \pm S.E.M.; $p=0.01$), but not at 7 months (1 ± 1 vs. 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, six months of treadmill step training alone failed to produce an improvement in the quantitative measures of hindlimb stepping analyzed.

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, 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 vs. 0 ± 0 ; $p=0.04$) and 7 (6 ± 3 vs. 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 analyzed 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 (see 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 vs. 0 ± 0 ; $p=0.02$) and 7 (9 ± 2 vs. 0 ± 0 ; $p<0.001$) months, and more steps than media-trained rats at 7 months (9 ± 2 vs. 1 ± 1 ; $p=0.005$, Fig. 1F, G, 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 vs. 2 ± 2 ; $p=0.27$) or 7 (9 ± 2 vs. 6 ± 3 ; $p=0.07$) months after transection (Fig. 1H, 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 vs. 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 , $p \leq 0.001$; media-trained, -3 ± 3 , $p \leq 0.001$; 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 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, 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 nonsteppers 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 volume that separates the GFAP-positive rostral and caudal stumps. We analyzed 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 ($1.8 \pm 0.33 \text{ mm}^3$ vs. $3.3 \pm 0.52 \text{ mm}^3$; $p=0.02$), suggesting that OEG cells induce a process conducive to tissue repair.

OEG transplantation promotes noradrenergic fiber 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 fibers correlates with improvements in motor function (Ramón-Cueto et al., 1998, 2000; López-Vales et al., 2006). Therefore, we analyzed 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, C). However, significantly more DBH-positive fibers per mm^3 of tissue reside in the GFAP-positive caudal stump in OEG- than in media-injected rats (Table 1, 14 ± 7 vs. 1 ± 0 ; $p=0.03$).

Additionally, DBH-positive fiber distribution patterns differ between media- and OEG-injected animals (Fig. 4D, 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 fibers sprout short distances in transected animals. Furthermore, while the difference in DBH-positive fiber density between the media- and OEG-injected animals within 250 μm of the caudal GFAP-negative zone was not statistically significant (10 ± 3 vs. 65 ± 37 ; $p=0.09$), the fiber density in the remainder of the block caudal to the lesion (>250 μm) was greater in OEG-injected animals (0 ± 0 vs. 7 ± 3 ; $p=0.01$). Thus, OEG transplantation is sufficient to promote substantial regeneration of DBH-positive fibers and this regeneration could contribute to recovery of motor function after a complete transection.

Prevalence of serotonergic fibers in the caudal stump following transection

Previous studies suggest that the presence of 5-HT-positive fibers 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, D) and axons spanning the GFAP-negative zone (Fig. 5E-G). Since media-injected rats did not contain serotonergic

fibers spanning the lesion site, the 5-HT-positive axons crossing the transection in OEG-treated rats most likely represent regeneration.

More detailed analyses within the area directly caudal to the lesion unexpectedly showed numerous 5-HT-positive fibers 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 fibers (Guest et al., 1997; Fouad et al., 2005) in the caudal stump following spinal transection. Similarly, we detected serotonergic fibers and occasional cell bodies in the caudal stump of media-injected animals (Fig. 5H). Furthermore, the 5-HT-positive fiber distribution was similar in the media- and OEG-injected groups, with 88% and 92% of the 5-HT-positive fibers, respectively, located more than 250 μm past the injury site (Fig. 5I, J). There was no difference in the 5-HT-positive fiber density below the transection site in media- and OEG-injected rats (18 ± 3 vs. 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 (Gomez-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 fibers 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 fibers 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 analyzed the presence of 5-HT-positive fibers in the caudal stump. While we found evidence of serotonergic fibers 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 fibers 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 fibers (Guest et al., 1997; Fouad et al., 2005) caudal to the transection site, the 5-HT-labeled fibers 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.

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For Peer Review

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Figure 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 ($***p\leq 0.001$) 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 to 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.

Figure 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 (\pm 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 non-stepping animals are significantly different from 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 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 $p = 0.11$).

Figure 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 .

Figure 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 fibers (arrows) extend into the GFAP-positive caudal spinal cord. (C) DBH-positive fibers (arrows) course into the GFAP-positive caudal stump in an OEG-injected animal, with some fibers extending more than 250 μm from the caudal border of the GFAP-negative zone (C corresponds to 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 fibers 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 .

Figure 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-labeled for GFAP and 5-HT immunoreactivity. 5-HT-positive axons (D, arrows) course into the caudal GFAP-

positive spinal cord (**D**, box in **C**). (**E-G**) 5-HT-positive fibers span the GFAP-negative scar of an OEG-untrained animal (boxed areas in **E** are enlarged in **F** and **G**). 5-HT-positive fibers (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-labeled 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 .

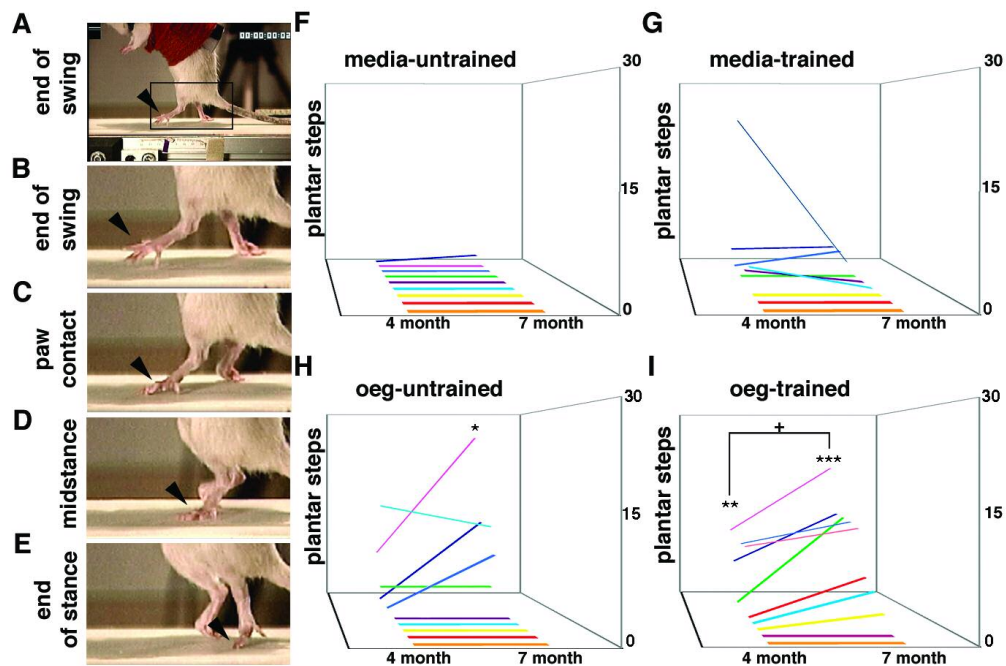
Table 1. The number of plantar steps each animal could perform is compared to the presence of 5-HT- and DBH-positive axons directly caudal to the transection site. Animals analyzed within each experimental group represent a range of stepping abilities. Each block containing the transection site yielded between 80 and 120, 25 μm -thick sagittal sections, and we analyzed 47-100% of the sections processed for 5-HT and DBH immunoreactivity. Fiber counts provide a density of fibers per cubic mm of tissue to account for size differences between the blocks examined.

Supplemental Figure 1. OEG primary and purified cultures. (**A**) Primary culture contains a variety of cell types. (**B**, **C**) Following immunopanning, purified cells

demonstrate the multipolar morphology of OEG (**B**) and express the p75 Nerve Growth Factor Receptor (**C**). Scale bar **A-C**, 25 μm .

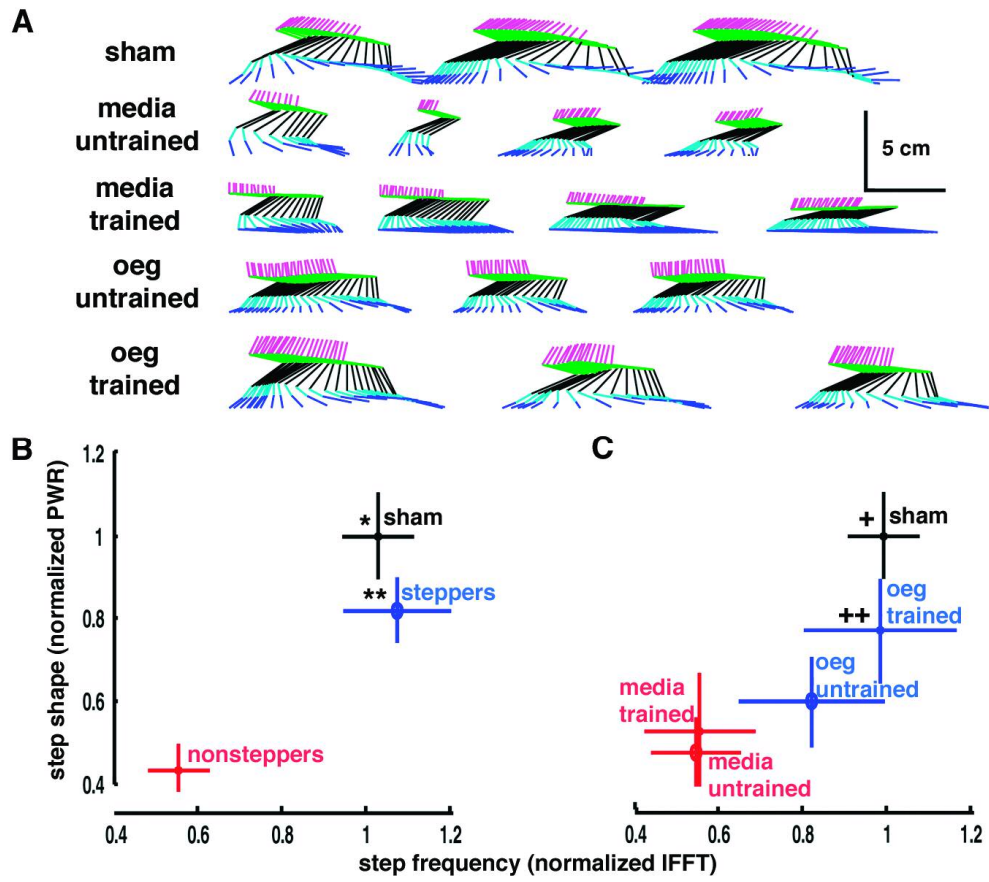
Supplemental Figure 2. The 25 μm -thick sagittal sections from the transection site of a media-untrained animal, also illustrated in Figs. 4D and 5I, are double-labeled for GFAP (amber-brown) and DBH (black) immunoreactivity. Sections are oriented with rostral towards the left and dorsal towards the top. Sections of the injury site are presented in order from **A-F** as illustrated in the drawing. (**A-F**) The rostral (left) and caudal (right) GFAP-positive spinal stumps are separated by a GFAP-negative scar (asterisks) in all sections examined, confirming a complete spinal cord transection. DBH-labeled axons (arrows) are detected in the rostral stump. Scale bar **A-F**, 400 μm .

Supplemental Figure 3. The 25 μm -thick sagittal sections from the injury site of an OEG-untrained rat, also seen in Figs. 4E and 5J, are labeled for both GFAP (amber-brown) and DBH (black, arrows) immunoreactivity. Sections are presented in order from **A-F** and oriented as in the drawing. (**A-F**) The rostral (left) and caudal (right) GFAP-positive spinal cord stumps are separated by GFAP-negative scar tissue (asterisks), confirming a complete spinal cord transection. The injury site in the OEG-untrained rat is more contiguous with the adjacent spinal cord stumps than sections from the media-untrained rat in Supplemental Figure 2. Scale bar **A-F**, 400 μm .



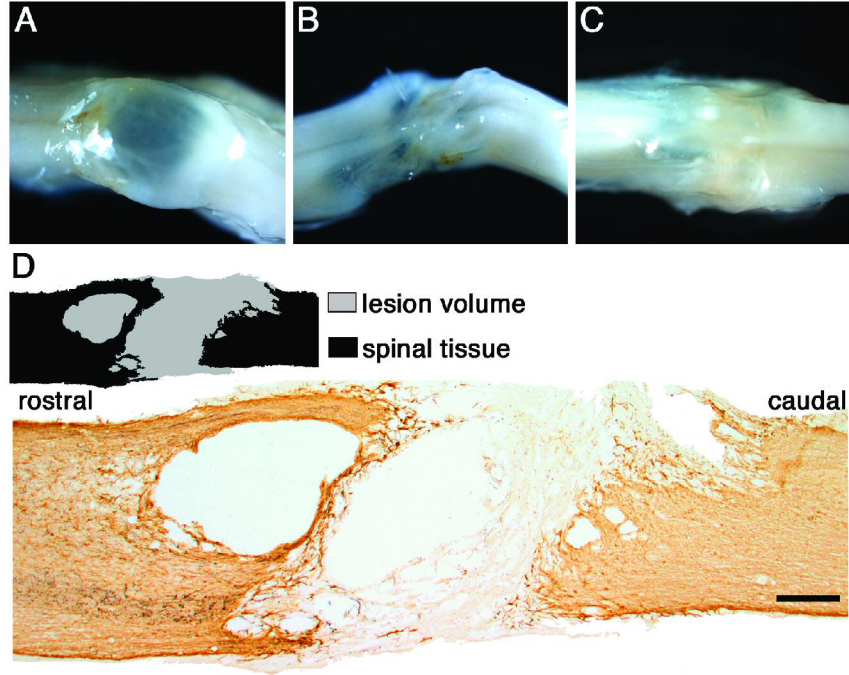
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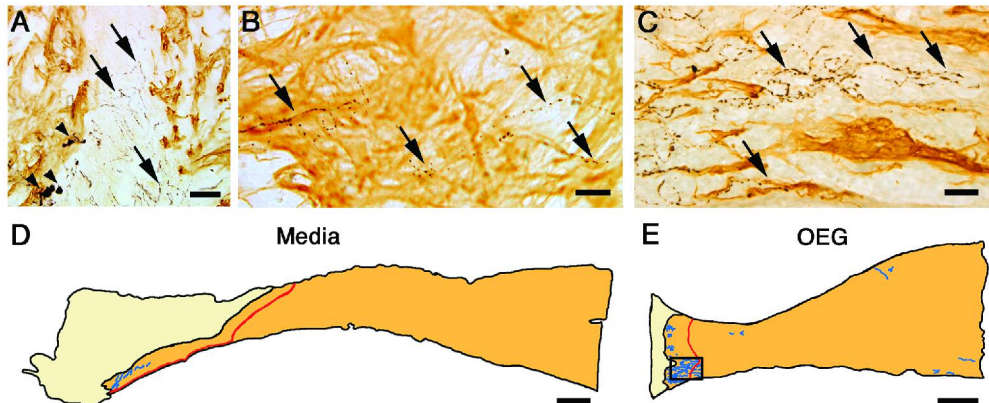
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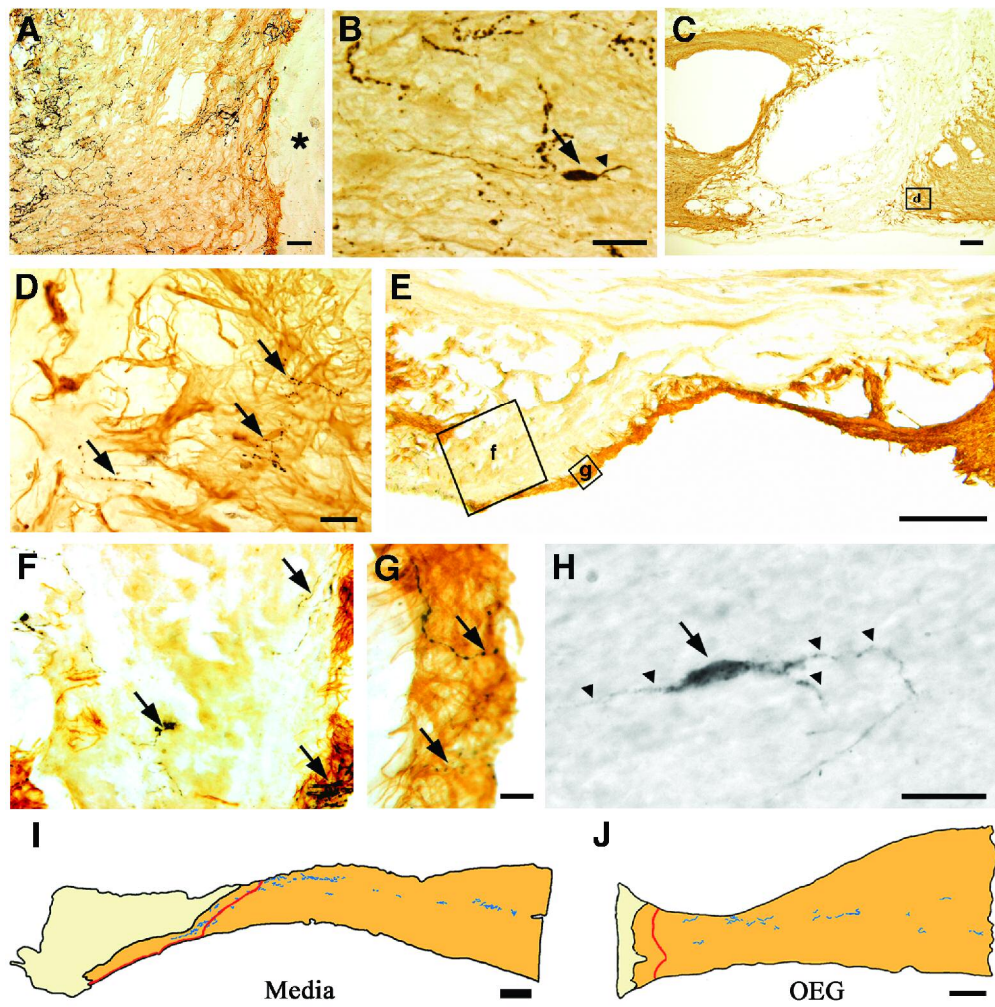
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view



176x75mm (300 x 300 DPI)

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176x178mm (300 x 300 DPI)

Table 1. Stepping performance and the presence of serotonergic and noradrenergic fibers

Experimental group	Plantar steps at 7 mo. (no.)	Amount of tissue block examined for 5-HT (%)	5-HT-positive fiber density (fibers/mm³)	Amount of tissue block examined for NA (%)	DBH-positive fiber density (fibers/mm³)
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#	n/a
OEG-untrained	8	n/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*

* p = 0.03

Two different OEG-untrained animals were analyzed for 5-HT- and DBH-positive fibers, respectively.
n/a, not available