METHODS

Study of the Efficiency of Transplantation of Human Neural Stem Cells to Rats with Spinal Trauma: the Use of Functional Load Tests and BBB Test

S. V. Lebedev, A. V. Karasev, V. P. Chekhonin, E. A. Savchenko, I. V. Viktorov, Yu. A. Chelyshev*, and G. F. Shaimardanova**

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 149, No. 3, pp. 355-360, March, 2010.
Original article submitted April 29, 2009.

Human ensheating neural stem cells of the olfactory epithelium were transplanted to adult male rats immediately after contusion trauma of the spinal cord at T9 level rostrally and caudally to the injury. Voluntary movements (by a 21-point BBB scale), rota-rod performance, and walking along a narrowing beam were monitored weekly over 60 days. In rats receiving cell transplantation, the mean BBB score significantly increased by 11% by the end of the experiment. The mean parameters of load tests also regularly surpassed the corresponding parameters in controls. The efficiency of transplantation (percent of animals with motor function recovery parameters surpassing the corresponding mean values in the control groups) was 62% by the state of voluntary motions, 37% by the rota-rod test, and 32% by the narrowing beam test. Morphometry revealed considerable shrinking of the zone of traumatic damage in the spinal cord and activation of posttraumatic remyelination in animals receiving transplantation of human neural stem cells.

Key Words: *contusion trauma of the spinal cord; human neural stem cells; transplantation; load tests; BBB test*

Fundamental studies at the tissue and cellular levels in the field of experimental cell therapy of spinal traumas attest to the existence of a considerable reparative potential in transplanted preparations of stem (progenitor) cells, but functional recovery of motor functions (organism level) was minimum [5,6,11]. This discrepancy can be explained by methodical problems, in particular by subjectivity of the method of semiquantitative evaluation of voluntary motions (BBB test) used in practically all studies; the error of the method according to authors' estimation is at least 1-2 points for the 21-point scale [4]. According to various reports, the maximum improvement of the motor functions by this scale after transplantation of cell preparations to animals with contusion traumas of the spinal cord (CTSC) did not exceed 2-3 points compared to the control [3,5-7,9-11,13]. The problem can be solved by using functional load tests allowing objective evaluation of motor function recovery, physical working capacity, and adaptive capacities of the whole organism [2].

Here we tested this methodological approach for preclinical evaluation of the efficiency of transplanta-

V. P. Serbskii State Research Center of Forensic and Social Psychiatry, Moscow; *Department of Histology, Kazan State Medical University; **Kazan Institute for Biochemistry and Biophysics, Kazan Research Center, Russian Academy of Sciences, Russia. ***Address for correspondence:*** lebedsv@yandex.ru. S. V. Lebedev

0007-4888/10/1493-0377

tion of neural stem cells (NSC) of human olfactory mucosa to rats in acute period of CTSC.

MATERIALS AND METHODS

Experiments were carried out on 53 outbred albino male rats weighing 300-350 g maintained under standard conditions of experimental biological clinics with free access to food and water.

The olfactory epithelium tissue was obtained in Neurovita hospital from patients with spinal traumas (the procedure was approved by Ethical committee of Russian State Medical University). Fragments of the olfactory mucosa (10×5 mm) were excised from the upper portion of the superior turbinate under local lidocaine anesthesia. The tissue sample was transferred to the laboratory in cold Ca^{2+},Mg^{2+}-free Hanks saline containing antibiotics and antimycotics (1:100, Gibco) not later than 2 h after isolation. The samples were repeatedly washed with the same buffer, blood vessels were removed, and the tissue was minced and incubated with trypsin and EDTA (0.25%) in 0.01 M phosphate buffered saline (pH 7.4) at 36.5°C for 40 min. The enzymes were blocked with DMEM (Gibco) containing 3% serum, the tissue was washed 3 times with balanced Hanks saline and dissociated by repeated pipetting in a nutrient medium. Nutrient media had the following composition: DMEM/F12 (Gibco), 10% FCS (Gibco), 2 mM glutamine (Gibco), 0.8% glucose, insulin+transferrin+sodium selenute (Gibco, 1:100), 20 mM HEPES, and growth factors (only for primary cultures): fibroblast growth factor (1 ng/ml; Sigma) and nerve growth factor (2 ng/ml; Sigma). The cell suspension was centrifuged at 1200 rpm for 7 min and the pellet was resuspended in the same medium. Only cell suspensions containing 90-95% viable cells were used for further culturing. Dissociated cells were cultured in 12-well plates on polylysine-coated substrate for 14 h. The medium was half-replaced twice a week. The primary culture after attaining a confluent state was harvested with trypsin-EDTA (0.25%), washed in Hanks balanced saline solution (HBSS), centrifuged, and resuspended in nutrient medium. The cell suspension was transferred to 25-cm flasks. Freely floating and attached to the substrate neurospheres were collected and dissociated by enzyme treatment. Suspension of neurosphere cells was cultured separately from ensheating glial cells, fibroblasts, and stromal (supportive) cells attached to the substrate. The cultures were thus subcultured 4 times. A portion of cells of the last passages was frozen and stored in liquid nitrogen.

The composition of NSC preparation was evaluated by immunocytochemical analysis of passage 3 neurospheres and subsequent 2-week culturing after defrosting. Cells of neurospheres primarily expressed nestin (neuronfilament protein) and B-tubulin after 3-fold washout of NSC in cold (4°C) Hanks saline, cell viability was evaluated by trypan blue exclusion test (this parameter was >95%) and a suspension with cells concentration of 100,000 cells per 1 ml Hanks saline was prepared. Sterile preparation of NSC was rapidly (within 5 min) delivered to the operation room, the time to transplantation did not exceed 1.5 h. During this period the preparation was stored at 4°C.

CTSC was modeled as described elsewhere [8]. The rats were intraperitoneally narcotized with 5% ketamine (120 mg/kg) and 0.5% seduxen (5 mg/kg). After laminectomy at Th9 level, the spine was rigidly fixed with metal clips by the spinous processes of vertebrae Th8 and Th10. Then, contusion of the spinal cord was modeled with a metal rod (2 mm in diameter, 10 mg) fixed in a stereotaxis micromanipulator and falling vertically from a height of 12.5 mm. The epicenter of the damage occurred along the central line of the spinal cord at the boundary with Th8. Then, Th8 laminectomy was carried out and NSC were transplanted into two points at the bondaries with Th7 and Th10: the cranial injection was made 0.3 mm to the left and the caudal one 0.3 mm to the right from the central line to avoid damage to the central channel of the spinal cord. We injected 750,000 cells in 10 μl Hanks medium in each point, *i.e.* 1.5 mln cells per rat (experimental group consisted of 30 rats). Controls (23 rats) received Hanks solution under the same conditions. Hamilton syringe with a steel needle (G29) connected to a micropump (Stoelting Co.) was used. The cells were injected to a depth of 1.2 mm, the rate of needle introduction was 10 μ/sec, the rate of injection was 1 μl/min. The needle was withdrawn 5 min after the end of transplantation. The operation wound was sutured layer-by-layer. Antibiotics were not administered. During postoperation days 1-3, voiding was forced by pressing the abdominal wall above the urinary bladder.

The rats were tested weekly at the same time of the day for 2 consecutive days: on day 1 voluntary movements by the BBB test and coordination disturbances by the narrowing beam test were assessed and on day 2 rota-rod performance was evaluated [2].

Morphological studies were carried out on a biomaterial taken 7, 30, and 60 days after trauma. The animals were narcotized (200 mg/kg ketamine intraperitoneally), perfused transcardially with cold (4°C) 4% paraformaldehyde in phosphate buffer (pH 7.4), and a fragment of spinal cord with the vertebrae (5 cm fragment length, 2.5 cm caudally and caudally from the lesion epicenter) was taken. After 12 h, the fragment was divided into 5 portions (1 cm each), which were numbered starting from the caudal one.

Fragments 1, 3, and 5 were postfixed in 2.5% glutaraldehyde and 1% OsO_4, dehydrated and embedded in epon 812. Fragments 2 and 4 were fixed in 10% neutral formalin, dehydrated, and embedded in paraffin. The area of pathological cavities and the amount of myelinated fibers at distances of 3 and 5 mm rostrally and caudally the epicenter of lesion were evaluated on semithin sections of the spinal cord prepared on an LKB III microtome and stained with methylene blue. The area of the white and gray matter at distances of 1 and 1.5 cm from the epicenter of the lesion was measured on paraffin sections. Morphometrical parameters were evaluated in 4 zones of the white matter: 1) ventromedial part of the anterior funiculus adjacent to the medial fissure on the right side; 2) the same on the left side; 3) lateral part of the lateral funiculus within the frontal plane passing through the central channel, right side; 4) the same on the left side.

For fluorescent visualization of the transplanted cells, 500,000 NSC stained with CFDA-SE (Invitrogen) in 5 μl Hanks medium were transplanted to 5 experimental rats into 2 above described points. The cells were labeled immediately before transplantation. To this end, NSC were incubated in 1 mM CFDA-SE in DMSO (1:100) on phosphate buffered saline for 15 min at 37°C and then in DMEM for 30 min at 37°C. The fragment of the spinal cord was taken on day 7 and frozen for preparing cryostat sections.

The data were processed statistically using Student's *t*, Wilcoxon–White, Mann-Whitney, χ^2 and Fisher exact tests.

RESULTS

Animal mortality was practically similar in the control and experimental groups (26 and 23%, respectively). Transplantation of NSC had little effect on body weight gain: body weight by the end of the experiment was 448±12 g in the control group and 449±15 g in the experimental group.

BBB testing revealed gradual partial spontaneous recovery of voluntary movements in rats of the control

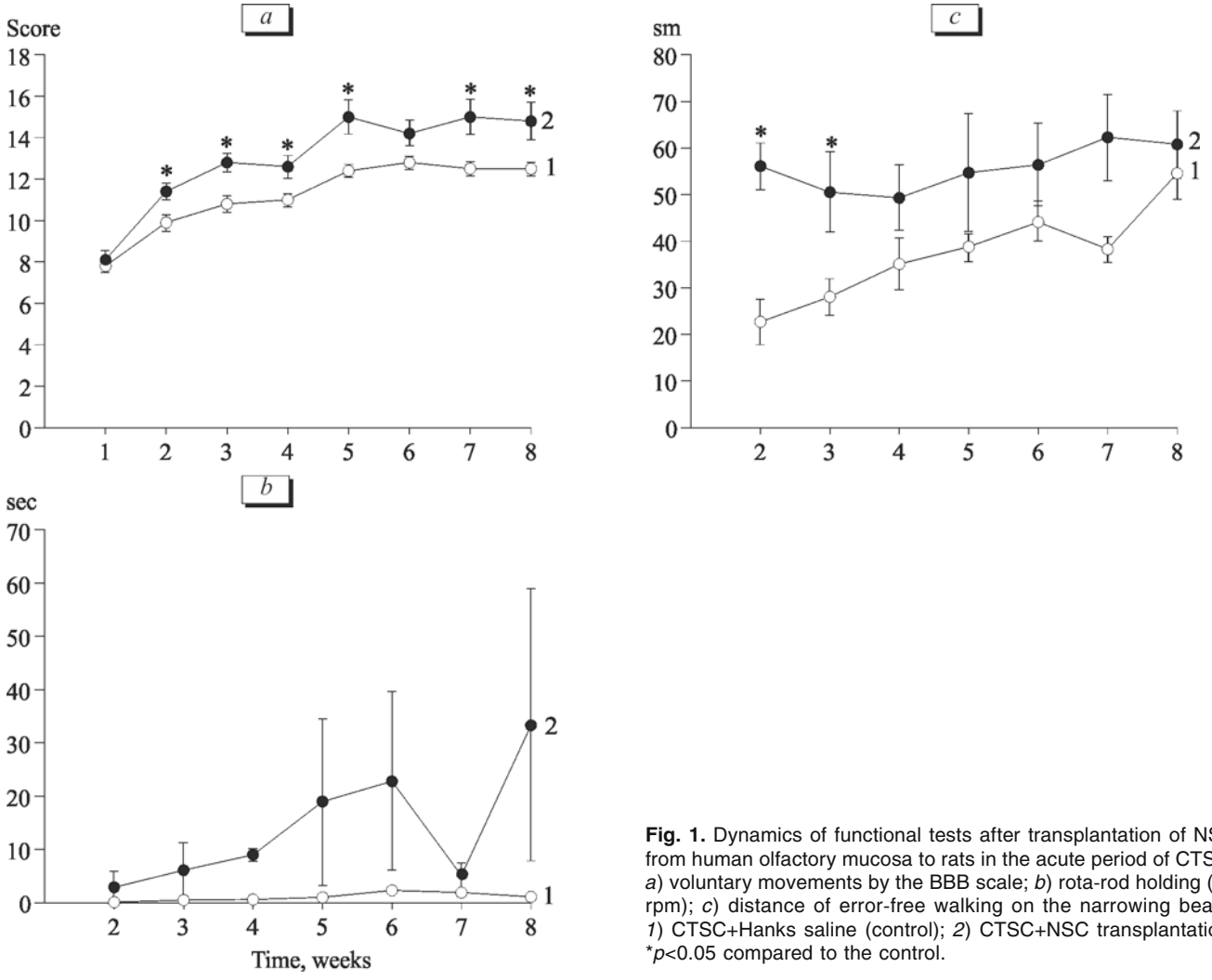

Fig. 1. Dynamics of functional tests after transplantation of NSC from human olfactory mucosa to rats in the acute period of CTSC. *a*) voluntary movements by the BBB scale; *b*) rota-rod holding (21 rpm); *c*) distance of error-free walking on the narrowing beam. *1*) CTSC+Hanks saline (control); *2*) CTSC+NSC transplantation. **p*<0.05 compared to the control.

group: BBB score increased from 7.8±0.2 one week after surgery to 12.5±0.3 by the end of the experiment (Fig. 1, *a*). In rats receiving NSC transplantation, the recovery of voluntary movements was more pronounced starting from the second week of monitoring. The mean increase in BBB score in the experimental group was 4.5±0.4 *vs.* 2.5±0.3 in the control ($p \leq 0.02$) after 30 days and 5.8±0.3 *vs*. 4.5±0.3 ($p \leq 0.002$) after 60 days.

The positive effect of NSC transplantation on recovery of voluntary movements was also confirmed by the analysis of relative parameters The percent of animals with BBB score ≥13 in the control and experimental groups after 30 days was 0 and 50% ($p \leq 0.05$), respectively; after 60 days the corresponding values were 25 and 84% ($p \leq 0.05$). In the experimental group, the percent of animals with maximum increase in this parameter (>5) 60 days after CTSC was also considerably higher than in the control: 100 and 38%, respectively ($p \leq 0.05$).

Transplantation of NSC increased the time of holding on the rotating rod of the rota-rod apparatus compared to the corresponding parameter in the control group throughout the monitoring period, but these changes were statistically insignificant (Fig. 1, *b*). The tendency to improvement of locomotor function after transplantation of NSC was confirmed by appreciable increase in the percent of rats capable of staying on the rotating rod (21 rpm) from 63% in the control group to 100% in the experimental group by the end of the experiment.

Transplantation of NSC also increased the mean distance of error-free walking along the narrowing beam (Fig. 1, *c*). Significant differences from the control in this parameter were observed only during the first 2 weeks of testing. This suggests that intraparenchymatous transplantation of NSC in the acute period of CTSC provides a short-term neuroprotective effect. Analysis of the relative number of animals walking more than 50 cm along the narrowing beam without

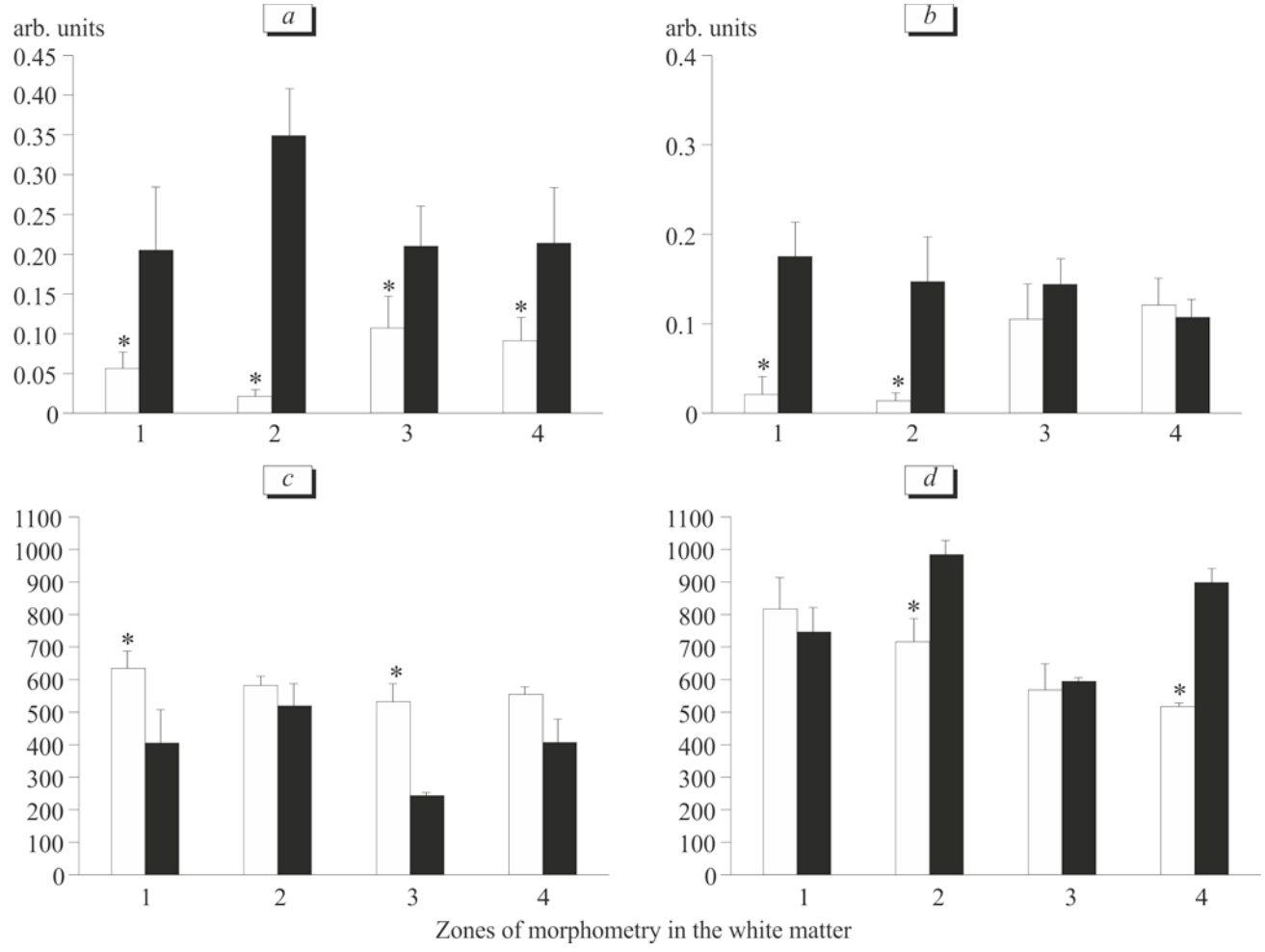

Fig. 2. Effect of NSC transplantation on nervous tissue recovery in rats with CTSC 30 days after surgery and transplantation. *a*, *b*) total area of cavities; *c*, *d*) amount of myelinated fibers; *a*, *c*) 3 mm caudally to the lesion epicenter; *b, d*) 5 mm. Open bars: experiment; dark bars: control. *$p<0.05$ compared to the control.

TABLE 1. Efficiency of NSC Transplantation in the Acute Period of CTSC in Rats (60 Days after Trauma)

Parameter	Percent of rats, %		Efficiency Δ%=%experiment-%control	*p*
	control	experiment		
BBB score ≥13	25	84	59	<0.05
BBB score ≥5	38	100	62	<0.05
Rota-rod holding at 21 rpm	63	100	37	0.1
The distance of error-free walking on the narrowing beam ≥50 cm	25	67	32	0.1

errors also attests to positive effect of NSC transplantation on hind limb coordination. In the experimental group, the percent of animals walking ≥50 cm along the narrowing beam without errors after 30 and 60 days surpassed the corresponding parameter in the control group by 5 and 2 times, respectively.

Calculation of NSC transplantation efficiency from comparison of the number of rats with considerable recovery of impaired functions (above the mean values in the control group) by the end of the experiment revealed considerable therapeutic effect by the parameters of voluntary movements (BBB scale), while improvement revealed by loading tests (rota-rod and narrowing beam) was statistically insignificant (Table 1).

Morphological analysis of CTSC zone showed that the character and the volume of damage to the gray and white matter in experimental rats corresponded to medium-severity CTSC (weight fall from a height of 12.5 mm) [4].

CFDA-SE-labeled NSC transplanted into the spinal cord were viable for at least 7 days. Their migration along the periphery of the lesion focus in the spinal cord was observed. Transplantation of NSC 5 mm rostrally and caudally to the lesion epicenter significantly prevented the formation of pathological cavities in the anterior funiculus of the white matter (Fig. 2, *b*). This phenomenon was most pronounced in sites adjacent to the epicenter of the lesion (Fig. 2, *a*), which definitely attests to the neuroprotective effect of NSC transplantation at the periphery of lesion site in the spinal cord tissue.

NSC transplantation also had a positive effect on the process of posttraumatic remyelination. The amount of myelin fibers in the lesion area (3 mm from the epicenter) in the experimental group surpassed the corresponding parameter in controls (Fig. 2, *c*). At the same time, this effect was not seen during the analysis of spinal cord sections at greater distance from the epicenter (5 mm, Fig. 2, *d*), which can be explained by additional trauma at this level caused by injection of the cell preparation at this site. Measuring of the area of the white and gray matter at a distance of 1 and 1.5 cm from the epicenter of the trauma revealed no significant differences between the control and experimental groups.

Low absolute (mean) values of locomotion recovery parameters in our experiments agree with the results of previous experiments on cell therapy of spinal cord injuries [3,7,10,11,13,15]. Insufficient level of functional reparation can be explained by peculiar tissue damage in spinal cord injuries: disruption of long nerve conduction pathways (corticospinal and rubrospinal tracts) and the absence of physiological (phylogenetic) mechanisms for restoring their continuity [12,14].

When evaluating the efficiency of cell therapy of CTSC we did not compare the means of functional recovery parameters, but the percent of animals with recovery parameters above the mean level in the control group by the end of the experiment (60 days after surgery). The efficiency of NSC transplantation into the spncal cord during the acute stage of CTSC was 62% by the BBB test (recovery of voluntary movements) and 32 and 37% by load tests narrowing beam and rota-rod, respectively. These results are an argument for planning clinical testing of NSC preparation in CTSC and at the same time put the question on the necessity of search for new methods of modulation of phylogenetic sanogenetic mechanisms involved in recovery of integral functions in spinal cord injuries.

The study was supported by the Russian Foundation for Basic Research (grant 08-04-01167 and 07-04-00746).

REFERENCES

1. I. V. Viktorov, E. A. Savchenko, and V. P. Chekhonin, *Kletochn. Tekhnol. Biol. Med.*, No. 4, 183-188 (2007).
2. S. V. Lebedev, S. V. Timofeev, A. V. Zharkov, *et al., Byull. Eksp. Biol. Med.*, **146**, No. 10, 471-476 (2008).
3. D. J. Barakat, S. M. Gaclani, S. R. Neravetla, *et al., Cell Transplant.*, **14**, No. 4, 225-240 (2005).

4. D. M. Basso, M. S. Beattie, and J. C. Bresnahan, *J. Neurotrauma*, **12**, No. 1, 1-21 (1995).
5. Q. L. Cao, Y. P. Zhang, R. M. Howard, *et al., Exp. Neurol.*, **167**, No. 1, 48-58 (2001).
6. B. J. Cummings, N. Uchida, S. J. Tamaki, *et al., Proc. Natl. Acad. Sci. USA*., **102**, No. 39, 14,069-14,074 (2005).
7. G. U. Enzmann, R. L. Benton, J. P. Woock, *et al., Exp. Neurol.*, **195**, No. 2, 293-304 (2005).
8. J. A. Gruner, *J. Neurotrauma*, 9, No. 2, 123-126 (1992).
9. M. J. Howard, S. Liu, F. Schottler, *et al., Somatosens Mot. Res*., **22**, Nos. 1-2, 37-44 (2005).
10. H. S. Keirstead, G. Nistor, G. Bernal, *et al., J. Neurosci.*, **25**, No. 19, 4694-4705 (2005).
11. H. J. Lee, K. S. Kim, I. H. Park*, et al., PLoS One*, **2**, No. 1, e156 (2007).
12. J. W. McDonald, D. Becker, T. F. Holekamp, *et al., J. Neurotrauma*, **21**, No. 4, 383-393 (2004).
13. G. W. Plant, C. L. Christensen, M. Oudega, and M. B. Bunge, *Ibid*, **20**, No. 1, 1-16 (2003).
14. P. J. Reier, *NeuroRx*., **1**, No. 4, 424-451 (2004).
15. T. Takami, M. Oudega, M. L. Bates, *et al., J. Neurosci.*, **22**, No. 15, 6670-6681 (2002).