

Engraftment of Serotonergic Precursors Enhances Locomotor Function and Attenuates Chronic Central Pain Behavior Following Spinal Hemisection Injury in the Rat

Bryan C. Hains,* Kathia M. Johnson,* David J. McAdoo,* Mary J. Eaton,† and Claire E. Hulsebosch*

*Department of Anatomy and Neurosciences and Marine Biomedical Institute, University of Texas Medical Branch, Galveston, Texas 77555-1069; and †The Miami Project to Cure Paralysis, University of Miami School of Medicine, 1095 NW 14th Terrace (R-48), Miami, Florida 33136

Received January 19, 2001; accepted June 14, 2001; published online August 24, 2001

Spinal cord injury (SCI) results in abnormal locomotor and pain syndromes in humans. T13 spinal hemisection in the rat results in development of permanent mechanical allodynia and thermal hyperalgesia partially due to interruption of descending inhibitory modulators such as serotonin (5-HT). We hypothesize that lumbar transplantation of nonmitotic cells that tonically secrete antinociceptive and trophic compounds will reduce the pain-like behavior and enhance locomotor recovery after SCI. We used RN46A-B14 cells, a conditionally immortalized (SV40tsTag) rat neuronal cell line derived from E13 raphe bioengineered to secrete both 5-HT and BDNF *in vitro* at both permissive (33°C) and nonpermissive (39°C) temperatures. Three groups ($n = 72$) of 30-day-old male Sprague-Dawley rats were spinally hemisectioned at T13 and allowed 4 weeks for adequate recovery of locomotor function and development of allodynia and hyperalgesia. Immunosuppressed animals received either lumbar RN46A-B14 ($n = 24$) or control RN46A-V1 ($n = 24$) empty-vector transplants or no cell ($n = 24$) transplant. HPLC analysis of media and CSF demonstrated increases of both *in vitro* and *in vivo* 5-HT levels at 28 days in RN46A-B14 animals. ELISA demonstrated BDNF secretion *in vitro* and *in vivo* by RN46A-B14 cells. Locomotor function (BBB scale) and nociceptive behaviors measured by paw withdrawals to von Frey filaments, radiant heat, and noxious pin stimuli were tested for 4 weeks posttransplant. Animals receiving RN46A-B14 cells demonstrated significantly improved locomotor function and reductions in both fore- and hindlimb mechanical allodynia and thermal hyperalgesia compared to controls receiving RN46A-V1 or no transplants. These effects were modulated by the 5-HT antagonist methysergide and reuptake inhibitor fluvoxamine. Bromodeoxyuridine and 5-HT immunoreactivity confirmed cell survival and graft location 4 weeks posttransplantation. These results support the therapeutic potential of bioengineered serotonin-secreting cell lines in reducing chronic central pain following spinal cord injury.

© 2001 Academic Press

Key Words: spinal cord injury; serotonin; brain-derived neurotrophic factor; transplantation; pain; locomotion; graft; transplant.

INTRODUCTION

Spinal cord injury (SCI) results in devastating losses of sensory and motor function below the level of the lesion as well as the existence of chronic pain syndromes in the majority of patients. A summarization of 13 epidemiological studies incorporating a total of 3107 spinally injured patients reveals that 64% suffer from chronic pain (18) which so severely compromises quality of life that suicide frequently ensues (12, 88). Central pain syndromes remain refractory to conventional analgesic treatments and demand novel therapeutic approaches. One such novel approach would be the transplantation of bioengineered cells that secrete substances that would attenuate the somatosensory behavioral deficits to an appropriate spinal site. In this study we used the RN46A-B14 cell line, which secretes both serotonin (5-HT) and brain-derived neurotrophic factor (BDNF).

Loss of 5-HT corresponds with severity of SCI (31), which correlates with loss of locomotor function (37, 107) and changes in nociceptive neural circuits (101, 104) that can provide a substrate for the persistence of chronic central pain syndromes. We have shown that after T13 hemisection injury, which interrupts descending serotonergic fibers, changes in serotonergic elements persist indefinitely. Lumbar dorsal horn analysis at both acute and chronic time points demonstrates bilateral reductions in 5-HT, which only partially recover, and increases in 5-HT transporter. In addition, behavioral indicators consistent with chronic central pain are reversed with intrathecal 5-HT (44). Replacement of 5-HT lost after injury should reverse chronic central pain-like behaviors by inhibition of no-

ciceptive neural circuits activated by either noxious (100) or formerly nonnoxious stimulation (17, 18).

BDNF is present or can be upregulated in reactive satellite cells in dorsal root ganglia (25, 49), spinal sensory neurons (3, 30), and superficial spinal dorsal horn (67) and ventral horn (49). It is suspected to play an important role in plasticity and survival of spinal neurons, as central administration has proven effective in preventing cell death (46) promoting regeneration after various types of injury (84, 103, 105) and altering synaptic efficiency (50, 62, 82). Relevant here, exogenous BDNF can modify the firing pattern of serotonergic neurons (15) and can promote sprouting of 5-HT axons (61, 64). Additionally, increased survival of axotomized corticospinal neurons by exogenously supplied BDNF has been demonstrated (35). BDNF has also been demonstrated to possess antinociceptive functions (19, 90).

Since analgesic intrathecal pump systems are associated with mechanical malfunction and infection of tunneled catheters and injection ports (65), and require periodic surgical maintenance and pump replacements, one-time transplantation of a bioengineered cell line is an attractive strategy in combating locomotor dysfunction and chronic pain following spinal injury. Our laboratory and others have demonstrated successes in transplant-induced reduction in pain-related behaviors with catecholamine- (42) as well as indolamine-secreting cells (29) for chronic periods of time. Furthermore, transplantation of embryonic raphe cells reinnervate (33) and activate locomotor circuitry after thoracic transection (32, 77). E13 rat raphe cells conditionally immortalized with temperature-sensitive mutants of the SV40 large T antigen (99) are effective in reducing pain-like behaviors in a model of peripheral neuropathic pain (29) but have not been explored in chronic spinal cord injury. Additionally, similarly bioengineered BDNF-secreting cells attenuate pain-like behaviors after sciatic nerve constriction (14). In a previous report we demonstrated that transplantation of RN46A-B14 cells after hemisection restores spinal serotonin, downregulates serotonin transporter, and increases BDNF tissue content in rat (43). In this paper, we tested whether transplantation of RN46A-B14 cells but not empty-vector control cells (RN46A-V1) would survive, secrete 5-HT and BDNF, and improve locomotor recovery while reducing pain-related behaviors following T13 spinal cord hemisection injury in rat.

MATERIALS AND METHODS

Subjects were male Sprague–Dawley rats, 100–125 g, obtained from Harlan Sprague–Dawley, Inc., housed with a 12:12-h light:dark cycle and fed *ad libitum*. All procedures involving rats were reviewed by the UTMB Animal Care and Use Committee and were consistent with the guidelines of the International Association for

the Study of Pain and the NIH Guide for the Care and Use of Laboratory Animals.

Spinal Cord Hemisection Injury

Rats ($n = 72$) were deeply anesthetized by intraperitoneal (ip) injection of sodium pentobarbital (40 mg/kg). The left side of the spinal cord was hemisected at T13 by the following procedure: following palpation of the dorsal surface to locate the rostral borders of the sacrum and dorsal spinous processes of the lower thoracic and lumbar vertebrae, the T11–T12 laminae were determined by locating the last rib, which attaches to the rostral end of the T13 vertebra. The surgical field was shaved and prepared with Betadine, and a longitudinal incision was made exposing several segments. A laminectomy was performed at the T11 vertebra, the lumbar spinal cord was identified with accompanying dorsal vessel, and the spinal cord was hemisected at spinal segment T13 with a No. 11 scalpel blade without damage to the posterior spinal blood vessel or branches. Muscle and fascia were sutured closed and skin closed with autoclips and animals were allowed to recover on a 36.5°C heating pad. Postoperative treatments included saline (1.0 cc sc) for rehydration and penicillin-G (0.35 ml/kg im) (Wyeth Laboratories, Philadelphia, PA) as a prophylactic antibiotic. Following surgery animals were maintained under the same preoperative conditions. The extent of the hemisection lesion, assessed histologically, was confined unilaterally and included the dorsal column, Lissauer's tract, lateral and ventral column systems, and gray matter.

Cell Culture and Transplantation

The RN46A-B14 and RN46A-V1 cell lines were grown at permissive temperature (33°C), in DMEM/F-12/10% FBS/250 μ g/ml G418/10 ml penicillin–streptomycin (Gibco, Rockville, MD) as described previously (29). The RN46A-V1 and RN46A-B14 cell lines were developed from embryonic day 13 rat medullary raphe nucleus, conditionally immortalized with the SV40tsTag, and transfected with a BDNF insert (14). Cells were proliferated in T25 flasks at 33°C to near confluence and/or pulse labeled for 3 days in the presence of 10 μ M bromodeoxyuridine (BrdU; Fischer), a nucleic acid commonly used to mark transplanted cell nuclei before collection for transplantation if they were to be subsequently examined for survival with anti-BrdU antibodies. Immediately before transplantation, cells were gently dissociated from T25 flasks with sterile 0.5 mM EDTA/Dulbecco's phosphate-buffered saline (DPBS; Gibco), pelleted by centrifugation, counted by trypan blue exclusion, and suspended in 10 ml Ca^{2+} – Mg^{2+} -free Hanks' buffered saline solution.

Animals ($n = 24$, RN46A-V1; $n = 24$, RN46A-B14) were deeply anesthetized by ip injection of sodium

pentobarbital (40 mg/kg). Following a partial laminectomy and small puncture of the dura, 10^6 cells were injected into the subarachnoid space of the lumbar dorsal spinal cord with a small length of polyethylene (PE10) tubing, by a dorsal/caudal entry into the dural puncture, at spinal segment L1, 28 days following T13 spinal hemisection injury. Twenty-four animals underwent sham transplantation. Muscle and fascia were sutured and the skin was closed with autoclips and animals were allowed to recover on a 36.5°C heating pad. Postoperative treatments included saline (1.0 cc) for rehydration and penicillin-G (0.35 ml/kg im) (Wyeth Laboratories) as a prophylactic antibiotic. To prevent acute host immune response, cyclosporine A (Novartis, 40 mg/kg daily) was given for 7 days postoperatively to all animals, including controls. Following surgery animals were maintained under the same preoperative conditions.

BDNF Enzyme-Linked Immunosorbent Assay (ELISA)

To directly measure BDNF concentrations *in vitro* or *in vivo*, from cell culture media, a specific sandwich ELISA was performed using a commercially available kit (BDNF E_{max} Immunoassay System; Promega, Madison, WI). Methods for quantification of mature BDNF protein by ELISA were adapted from Cejas *et al.* (33). Microtiter 96-well flat-bottom ELISA plates (Nunc MaxiSorp) were coated with 100 μ l/well anti-BDNF monoclonal antibody in carbonate buffer (pH 9.7). After overnight incubation at 4°C, plates were blocked to avoid nonspecific binding with 200 μ l/well of Blocking and Sample Buffer for 1 h at room temperature. After the wells were washed with TBST wash buffer, 100 μ l/well of serum-free medium, stock medium, or culture medium from confluent RN46A-V1 ($n = 5$) or RN46A-B14 ($n = 5$) T25 flasks (grown for 3 days at 33°C) was added. In addition, 100 μ l/well of either control CSF ($n = 7$) or CSF from animals receiving either RN46A-V1 ($n = 7$) or RN46A-B14 ($n = 7$) cell transplants and undergoing behavioral testing was added. Samples were loaded in triplicate. Serially diluted BDNF standard was used to generate a standard curve from 7.8 to 500 pg/ml. The plates were then incubated for 2 h with 100 μ l/well anti-BDNF polyclonal antibody and then incubated for 1 h with anti-IgY horseradish peroxidase conjugate in Blocking and Sample Buffer (1:2000) at room temperature. Color development was achieved with a mixture of peroxidase substrate in TMB, 100 μ l/well. After approximately 3 min, the reaction was stopped by the addition of 100 μ l/well 1 M phosphoric acid, and optical absorbance was recorded at 450 nm with a microplate reader. The average of triplicate data was obtained and protein concentrations were determined from the standard curve.

5-Hydroxyindoleacetic Acid (5-HIAA) High-Pressure Liquid Chromatography (HPLC)

5-HT is oxidized rapidly to 5-hydroxyindoleacetic acid, which was measured from cell culture and CSF. Twenty-eight days posttransplant, HPLC with electrochemical detection was performed. Stock media ($n = 5$), serum-free media ($n = 5$), and media of confluent RN46A-V1 ($n = 5$) or RN46A-B14 ($n = 5$) cell cultures (grown for 3 days at 33°C) or CSF obtained 28 days posttransplant from rats undergoing behavioral testing that received either RN46A-V1 ($n = 7$) or control RN46A-B14 cell transplants ($n = 7$) were analyzed. In the latter, deep anesthesia was induced with sodium pentobarbital (40 mg/kg ip) and a laminectomy and puncture were made at the atlanto-occipital membrane, and polyethylene PE10 tubing attached to a 250- μ l Hamilton syringe was threaded approximately 2 mm into the subdural space. CSF perfusate was collected through the catheter assembly and samples were then stored frozen at -70°C for later analysis. 5-HIAA levels were analyzed on a chromatograph consisting of a Beckman Model 118 solvent module, a Beckman System Gold data system, and an ESA Coulochem II electrochemical detector. A 150-mm-long, 3-mm diameter ESA C18 column was used. The mobile phase consisted of 75 mM NaH₂PO₄, 1.7 mM 1-octanesulfonic acid, 100 ml/L triethylamine, 25 mM EDTA, and 10% acetonitrile dissolved in 0.1-mm Millipore water at pH 3.22. Concentrations of 5-HIAA were calculated from relative peak heights to known standard sample concentrations.

Immunohistochemistry

Cells were grown in eight-well Costar culture slides, fixed in 4% paraformaldehyde, washed with DPBS, and treated with 200 μ l/well of permeabilization buffer (Triton X-100) for 30 min. Cells were then incubated in either anti-5-HT (Chemicon 1:5000; Temecula, CA) or anti-BDNF (Chemicon, 1:1000) antibodies overnight at 4°C. Cells were rinsed and incubated with anti-IgG Alexa Green 488 or Texas red (Molecular Probes, Eugene, OR) secondary fluorescent reporter in permeabilization buffer (1:150) for 1 h. After reactions were completed, slides were coverslipped using Vector No-Fade mounting medium.

At 28 days posttransplant following behavioral assessments, animals receiving cell transplants ($n = 10$, RN46A-V1; $n = 10$, RN46A-B14) were deeply anesthetized with sodium pentobarbital and perfused intracardially with heparinized 0.9% saline followed by 4% cold buffered paraformaldehyde in PBS. After perfusion, spinal cords were postfixed for 24 h and stained for either BrdU or 5-HT to assess transplant integrity and location, with positive immunoreactivity indicating transplant survival and 5-HT synthesis. After removal from the vertebral column, spinal cord samples

spanning the L1–L5 segments were postfixed for 24 h, at 4°C in 30% sucrose. Sections were stained intact for surface BrdU and 5-HT and, following preincubation in normal goat serum, reacted for 12 h at room temperature with anti-BrdU (Becton-Dickinson 1:500; Franklin Lakes, NJ) or anti-5-HT (Chemicon 1:5000). To test for host immune response to transplanted tissue, a monoclonal antibody against rat monocytes/macrophages (ED-1; Chemicon 1:400) was used. Additionally, to test for cell division and proliferation, an antibody against proliferating cell nuclear antigen (PCNA; Santa Cruz Biotechnology 1:400; Santa Cruz, CA) was used. Secondary antibody, appropriate biotinylated IgG (Vector Laboratories, Burlingame, CA), was applied for 90 min at room temperature followed by ABC–HRP Vector kit reagents. The reaction was completed and enhanced using diaminobenzidine with 0.01% H₂O₂. Tissue was then cleared and mounted in Permount (Fisher, Fair Lawn, NJ), after antibody staining for visualization and photomicroscopy using an Olympus microscope coupled to a Spot-2 Megapixel camera. Controls included preabsorbed sections and absence or presence of serum, or primary or secondary antibody, or ABC reagents.

BBB Locomotor Scoring

Locomotor function was observed three times weekly for 28 days following hemisection injury and recorded using the Basso, Beattie, and Bresnahan (BBB) Locomotor Rating Scale (6), which examines motor recovery to ensure reliability of somatosensory testing. Briefly, the BBB is a 21-point ordinal scale ranging from 0, which is no discernable hindlimb movement, to 21, which is consistent and coordinated gait with parallel paw placement of the hindlimb and consistent trunk stability. Scores from 0 to 7 rank the early phase of recovery with return of isolated movements of three joints (hip, knee, ankle); scores from 8 to 13 describe the intermediate recovery phase with return of paw placement, stepping, and forelimb–hindlimb coordination; and scores from 14 to 21 rank the late phase of recovery with return of toe clearance during the step phase, predominant paw position, trunk stability, and tail position. The scores were tabulated and considered to be an indicator of motor recovery after spinal cord hemisection. Animals demonstrating loss of locomotion in both hindlimbs, indicating bilateral corticospinal tract transection, or displaying signs of autophagia were excluded from the study (3 animals total in this study, resulting in the 72 animals reported above).

Somatosensory Behavioral Tests

Behavioral testing was done in a blinded fashion to surgical status or drug used. Preliminary experiments show that when spinally hemisected rats are compared to spinally hemisected rats with catheters in place,

there are no significant differences in response profiles in behavioral tests described below. Animals were tested preoperatively to establish baseline responses and tested 28 days postinjury. Behavioral testing (excluding BBB) was performed for both fore- and hindlimbs, regions innervated by C8 contributions of the median and ulnar nerves to the ventral forepaw and L5 contributions of the medial and lateral plantar nerves to the ventral hindpaw.

Intrathecal Drug Administration

In a separate group of animals, at the time of transplantation ($n = 30$), a 32-gauge intrathecal (i.t.) catheter (Micor, Inc., Allison Park, PA) was premeasured, filled with lactated PBS (pH 7.4), and inserted through a slit in the atlanto-occipital membrane caudally down the vertebral column to the T13 spinal subarachnoid space and sutured in place, and an 8-mm leader was left free to exit the skin. The distal free end of the catheter was heat sealed to prevent infection. One week after placement when anti-nociception was most robust, animals were tested for mechanical allodynia and thermal hyperalgesia. Immediately following this baseline measure, the 5-HT antagonist methysergide maleate (125 µg/kg; RBI, Natick, MA) or selective 5-HT reuptake inhibitor fluvoxamine maleate (75 µg/kg; Tocris, Ballwin, MO) was administered to examine the role of 5-HT based on literature dosing concentrations. Drug delivery was made by first applying suction through the i.t. catheter with a Hamilton syringe until cerebrospinal fluid was visible in the externalized part of the catheter to ensure patency. Catheters were then flushed with a small volume of sterile PBS (1–2 µl), and drugs were administered in 25 µl normal PBS, followed by 10 µl PBS flush injected into the subarachnoid space at T13. Prior to behavioral testing, drug efficacy onset and offset points were determined and behavioral assays for these drugs began 30 min after injection. Behavioral testing was performed within this predetermined window of efficacy. At least 24 h was allowed between each drug trial to allow drug washout, at which time all rats were tested for recovery to pre-drug responses to test for possible desensitization or sensitization. Postdrug tests were not statistically different compared to predrug trials or vehicle injections.

Mechanical Allodynia—von Frey

A blinded observer performed behavioral tests examining mechanical allodynia, pre- and postoperatively, for both forelimbs and hindlimbs. Prior to testing, all animals were environmentally acclimated to the clear Plexiglass cubicle testing apparatus (8 × 8 × 18 cm) for 4 h daily for 3 days. Following acclimation, preoperative testing began 3 days prior to injury to establish both individual and group baseline behaviors. Tests were performed postoperatively at weekly intervals to

ensure development of chronic pain. Since no significant side-to-side differences were found, data from each limb were collapsed into combined fore- or hind-limb scores for ease of comparison.

Mechanical allodynia of the glabrous skin of the paw was quantified by measuring number of brisk paw withdrawals, accompanied by active attention of the rat to the stimulus by head turning and stimulus attack, body postural changes, etc., in response to mechanical stimuli (16). The inclusion of these complex behaviors excludes simple hyperreflexia, which is a segmental response. Low- and medium-intensity stimuli were delivered by von Frey filaments (Stoelting, Wood Dale, IL) with bending forces of 4.78 and 9.96 mN. High-intensity von Frey filaments with a bending force of 204.1 mN were used. In addition, a normally noxious pinprick stimulus was also used to control for sedative or analgesic effects.

To perform these tests, rats are placed inside the Plexiglas boxes on an elevated, fine metal screen and acclimated for 60 min prior to testing. The von Frey filament was applied through the mesh to the ventral (plantar) surface of the glabrous skin of the paw for each limb. A single trial consisted of 10 applications of von Frey filament, applied once every 3 to 4 s. The mean occurrence of paw withdrawals in each of the trials was expressed as the number of responses out of 10. Supraspinal responses such as whole body changes, writhing, and vocalizations accompanied the paw withdrawals. Data were analyzed and graphed as group data and compared to baseline and at 28 days with no or various drug concentrations for between-group comparisons.

Thermal Hyperalgesia—Heat Lamp

Thermal hyperalgesia was measured by latency of paw withdrawal in response to a radiant heat source as previously described by Bennett and Xie (7) and Hargreaves *et al.* (45), using a newer method with constant glass temperature (30°C) for nontested limbs (27). Animals were placed in Plexiglas boxes on an elevated glass plate under which a radiant heat source (4.7 A) was applied that directed a beam of light onto the plantar surface of each paw through the glass plate. The light beam is turned off automatically by a photocell upon limb-lift, allowing the measurement of time between start of the stimulus and paw withdrawal (paw withdrawal latency). Supraspinal responses such as whole-body changes, writhing, and vocalizations accompanied these responses. Five minutes was allowed between each trial and three trials were averaged for each limb, which were then combined. Since no significant side-to-side differences were found, data from each limb were collapsed into combined fore- or hind-limb scores for ease of comparison. These data were analyzed and graphed as group data in which the base-

line is displayed as zero, and the data were compared for significance by between-group comparisons.

Statistical Analysis

All statistical tests were evaluated at the α level of significance of 0.05, by two-tailed analyses using parametric tests since the data passed normality and equal variance tests. Data from these procedures were tested for statistical significance using ANOVA and Friedman's rank test using one-way repeated measures, followed by tests of factors including pair-wise comparisons where appropriate with either the paired Student *t* test (comparisons of behavioral test results before and after hemisection surgery or comparison before and after treatment within each animal) or the two-sample Student *t* test (between-group) comparisons. Correlations of behavioral outcomes with different doses were tested by Pearson product moment correlation coefficients. All data management and statistical analyses were performed using Jandel SigmaStat (v1.0). All values were graphed using Jandel SigmaPlot (v5.0) as means \pm standard deviation (SD).

RESULTS

5-HIAA HPLC

HPLC analysis of cell culture media and CSF from transplanted animals revealed that concentrations of 5-HIAA, the oxidized metabolite of 5-HT, were increased only in the presence of RN46A-B14 cells (Fig. 1). *In vitro* confluent media assays demonstrated 5-HIAA levels of 8.02 ± 1.28 and 8.58 ± 1.94 ng/ml for RN46A-V1 and media-alone cultures, while RN46A-B14 cells produced significantly increased ($P < 0.05$) levels, 54.2 ± 4.12 ng/ml. *In vivo*, 28 days after transplantation, CSF concentrations were significantly ($P < 0.05$) increased in animals that received RN46A-B14 cells at 52.8 ± 5.87 ng/ml, compared to 24.5 ± 3.22 and 25.5 ± 2.81 ng/ml for RN46A-V1 and sham control animals. These increases, under both *in vitro* and *in vivo* conditions, represent six- and twofold increases, respectively.

BDNF ELISA

Cell culture media and CSF levels of BDNF, as determined by ELISA, are shown in Fig. 2. *In vitro* BDNF levels in culture media of confluent T25 flasks were significantly ($P < 0.05$) elevated in the RN46A-B14 groups compared to media from RN46A-V1 groups grown at 33°C. Levels in DMEM/F-12 base media and DMEM/F-12 plus fetal bovine serum (DFFGP) were 2.90 ± 1.22 and 10.0 ± 1.58 pg/ml, respectively. DFFGP of confluent (3×10^6 cells) cultures of

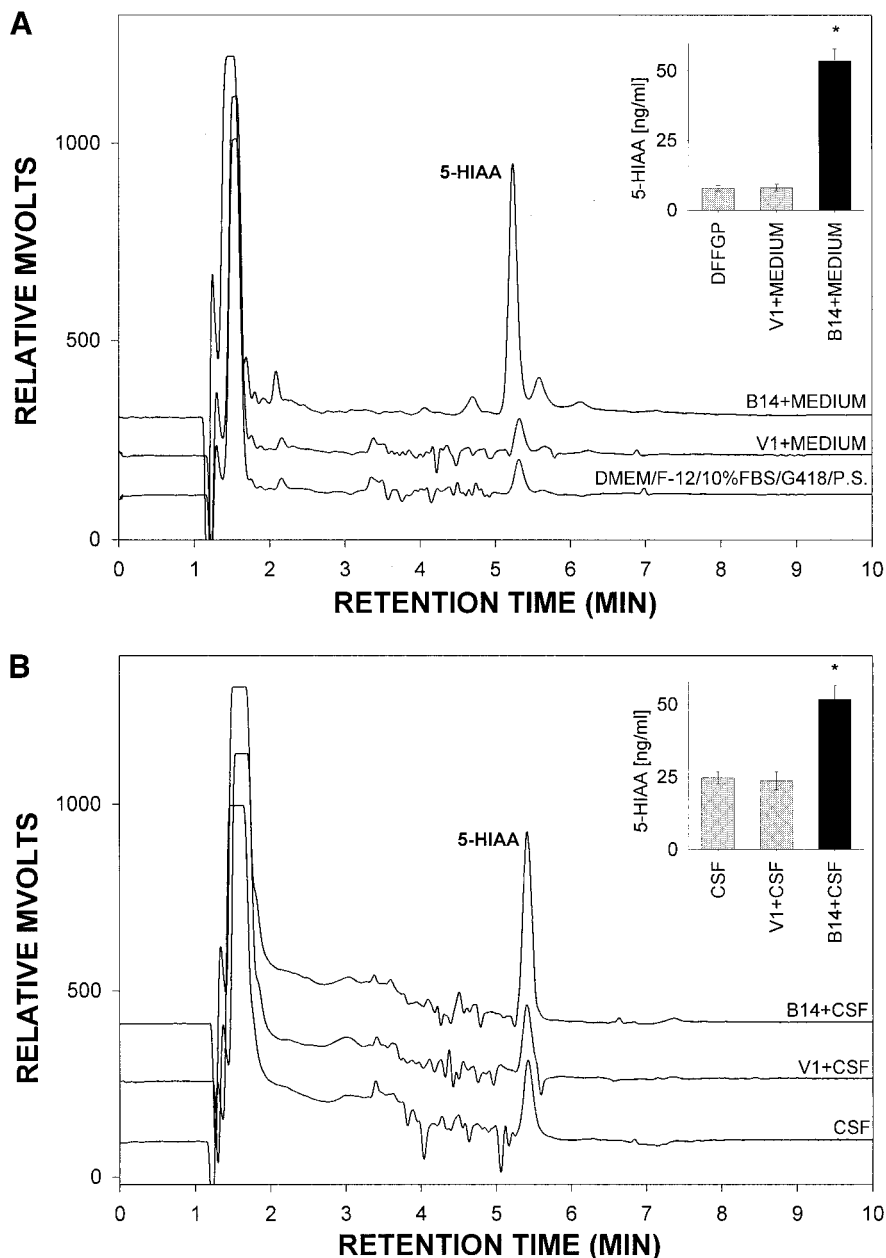


FIG. 1. HPLC analysis of 5-HIAA content in culture media (A) of confluent (3×10^6 cells) RN46A-B14 or RN46A-V1 cells grown at permissive temperature (33°C) in medium (DMEM/F-12/10% FBS/G418/PS) or medium-alone conditions and in cerebrospinal fluid (B) 28 days after L1 transplantation of either RN46A-B14 and RN46A-V1 cell line following injury or no transplant (37°C). In both cases, RN46A-B14 cells secreted significantly ($*P < 0.05$) higher levels of 5-HT revealed by a 5-HIAA peak at 5.38 min in both *in vitro* and *in vivo* assays compared to RN46A-V1 and control groups. Quantifications of samples (ng/ml; mean \pm SD) are shown as insets.

RN46A-V1 and RN46A-B14 cells were 8.50 ± 2.54 and 347.6 ± 26.8 pg/ml, respectively. Thus, serum contributed slightly to the increased levels of BDNF *in vitro*, but media of RN46A-B14 cells contained a 43 \times increase compared to RN46A-V1 media.

CSF from control (hemisected) animals contained 9.40 ± 2.10 pg/ml BDNF, which was not significantly different from that of animals receiving RN46A-V1 cell transplants (8.81 ± 1.58) at a nonpermissive temper-

ature (37°C). Twenty-eight days following transplantation of RN46A-B14 cells, CSF BDNF content was 52.1 ± 5.10 pg/ml. The distinction between RN46A-V1 and RN46A-B14 represents a sixfold increase in RN46A-B14 CSF values. It can therefore be concluded that BDNF produced by RN46A-V1 cells is not significantly different from normal media or CSF levels and that RN46A-B14 cells produce and secrete BDNF in levels that are significantly increased.

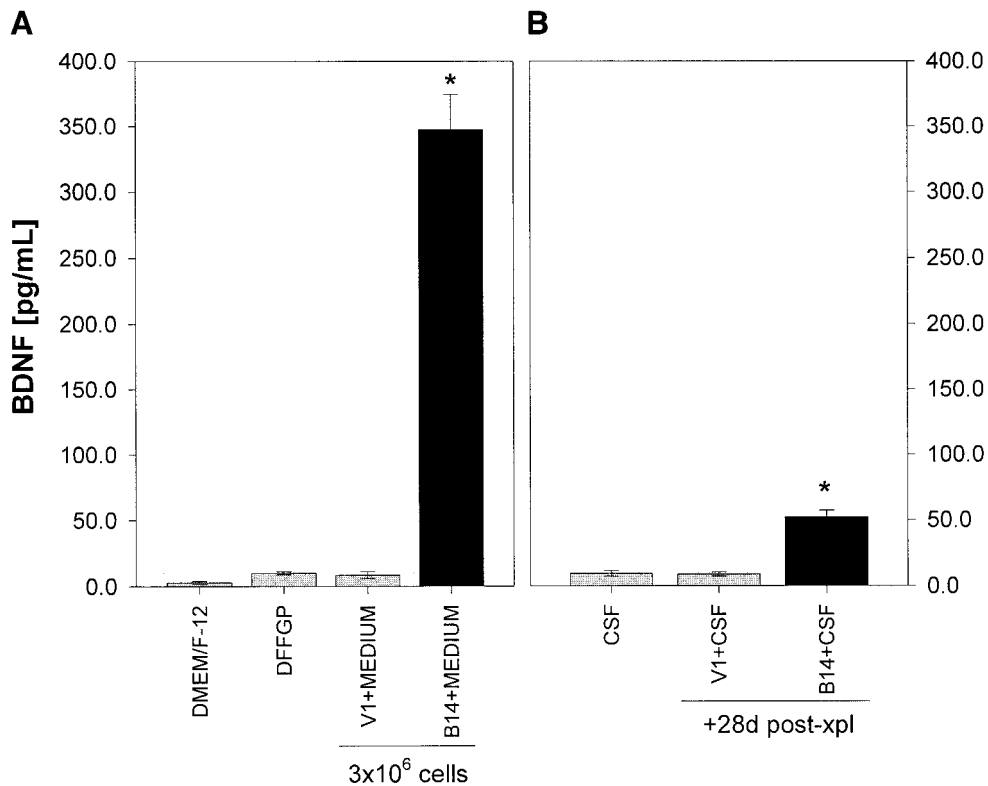


FIG. 2. ELISA determination of BDNF (pg/ml; mean \pm SD) content in confluent (3×10^6 cells) culture media (A) of RN46A-B14 and RN46A-V1 cells grown at permissive temperature (33°C) in DMEM/F-12/10% FBS/G418/PS and in cerebrospinal fluid (B) 28 days after L1 transplantation of either RN46A-B14 and RN46A-V1 cell lines following injury or no transplant (37°C). In both systems, RN46A-B14 but not RN46A-V1 cells secreted significantly ($*P < 0.05$) elevated levels of BDNF compared to normal conditions.

Recovery of Locomotor Function

Immediately upon emerging from anesthesia, hemisectioned animals displayed loss of ipsilateral hindlimb function as indicated by the BBB scores for each of the three groups (mean \pm SD): 1.75 ± 1.75 , 0.40 ± 0.21 , and 2.6 ± 1.54 for RN46A-B14, RN46A-V1, and sham groups, respectively (Fig. 3). By day 28, rats gradually regained use of their ipsilateral hindlimbs and attained scores of 14.2 ± 1.49 , 13.8 ± 2.77 , and 13.4 ± 1.53 , for the same three groups, respectively. Initially animals showed little or no movement in any of the joints of the affected limb but functional recovery began with movement of the hip, followed by the knee and finally the ankle joint. At 28 days, animals were transplanted with either RN46A-B14 or RN46A-V1 or no cells. By 42 days posttransplant, the BBB score for animals receiving RN46A-B14 cell transplants was 16.9 ± 1.08 , while animals that received vector-only cells and no transplant were 14.0 ± 1.12 and 14.4 ± 1.15 , respectively. By this time point, RN46A-B14 animals demonstrated significantly ($P < 0.05$) improved locomotor function compared to the two other groups. Forelimb and contralateral hindlimb motor scores were unaffected by the T13 hemisection and scored 21 throughout the period of behavioral testing.

Immunostaining

In vitro staining experiments demonstrated that RN46A-B14 cells, but not RN46A-V1, produced both BDNF and 5-HT (Fig. 4). This figure also demonstrates a characteristic morphology of the cell line demonstrating a mature neuronal phenotype after proliferation *in vitro*.

Twenty-eight days after transplantation, animals that received both RN46A-V1 and RN46A-B14 transplants demonstrated positive BrdU staining at low magnification on the dorsal aspect of the spinal cord, although in the RN46A-B14 group staining was more robust (Fig. 5). This is likely due to the requirement of BDNF for survival (only the RN46A-B14 cells exhibit autocrine production). At 28 days posttransplantation, 5-HT-positive staining was grossly observed in low-magnification views of the dorsal surface of the spinal cord at L1 in RN46A-B14-receiving animals but not in any other groups. Transplanted cells were found in aggregates or dispersed singly over the spinal cord surface; no cells were found more than two segments from the original transplant site.

To test for posttransplantation proliferation, PCNA immunocytochemistry was done on several samples. There were no PCNA-positive cells present in any sam-

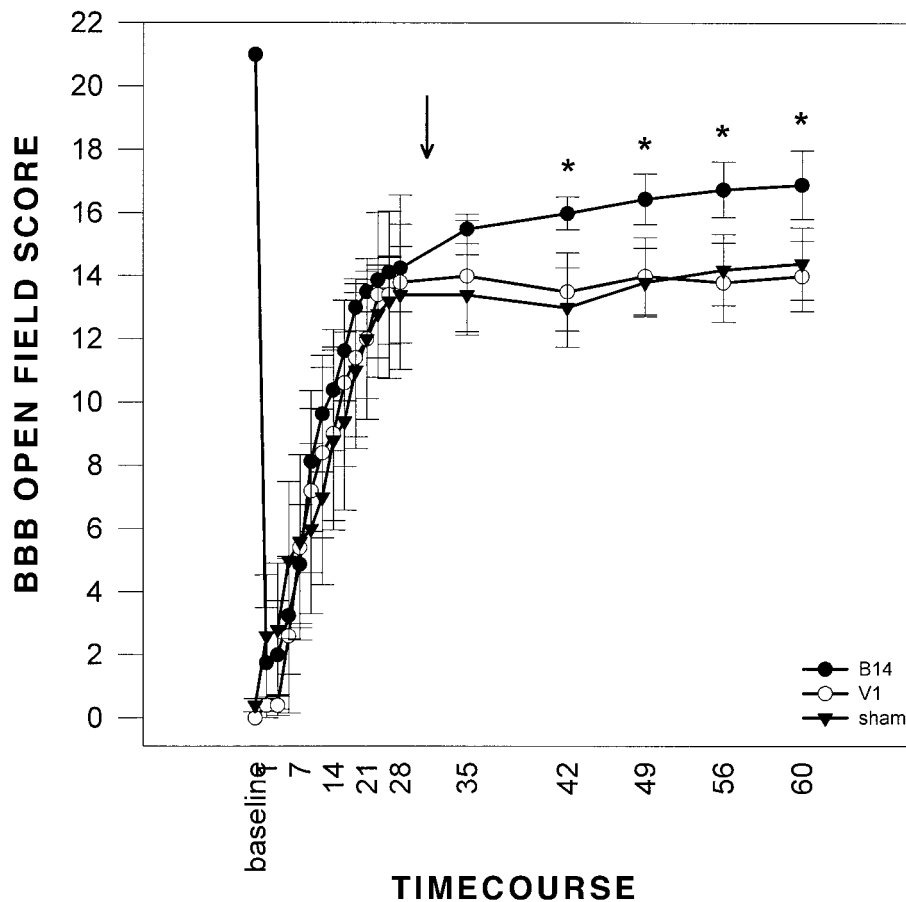


FIG. 3. BBB analysis of locomotor function following T13 hemisection and L1 transplantation (arrow) of sham, RN46A-V1, and RN46A-B14 cells displayed as mean \pm SD scores. Twenty-one represents normal locomotion, while zero represents no observable limb movement or weight support. Prior to transplant all animals recovered significant function, but by 42 days RN46A-B14-receiving animals demonstrated significantly ($*P < 0.05$) improved performance over all other groups, which by 60 days scored 16.9 ± 1.08 , while animals that received vector-only cells and no transplant scored 14.0 ± 1.12 and 14.4 ± 1.15 , respectively.

ple. To test for transplant-induced host/graft reactivity, samples were stained for monocytes and macrophages with ED-1. No ED-1-positive cells were found in any sample.

Behavioral Testing and Drug Administration

Mechanical allodynia. The numbers of paw withdrawals accompanied by complex aversion behaviors to low-threshold mechanical stimuli produced by 4.79, 9.96, and 204.1 mN von Frey filaments are shown as combined mean \pm SD numbers of paw withdrawals for the combined ipsilateral and contralateral limbs for both fore- and hindlimbs (Fig. 6). After 28 days, spinal hemisection produced statistically significant ($P < 0.05$) increases in paw-withdrawal frequencies of 3.62 ± 0.21 , 3.50 ± 0.37 , and 3.53 ± 0.46 for RN46A-B14, RN46A-V1, and sham groups in forelimbs receiving low-intensity 4.79-mN von Frey filament stimulation, from a baseline of 0.08. Hindlimbs also developed mechanical allodynia at the 4.79-mN strength, increas-

ing from 0.26 to 3.23 ± 0.18 , 3.12 ± 0.37 , and 3.15 ± 0.21 in RN46A-B14, RN46A-V1, and sham groups, respectively, which represents a statistically significant increase for all three groups. After receiving RN46A-B14, RN46A-V1, or no transplant, beginning at 36 days significant reductions in paw withdrawals were observed in animals that received RN46A-B14 cell transplants compared to RN46A-V1 and nontransplanted animals, and frequencies of paw withdrawal remained significantly decreased 28 days following transplantation. In forelimbs, at 4.79-mN strength, the RN46A-B14 group withdrew 2.10 ± 0.28 times, while RN46A-V1 and sham groups withdrew their paws 3.91 ± 0.25 and 3.42 ± 0.30 times, respectively. Similarly in hindlimbs, RN46A-B14 animals scored 1.21 ± 0.21 compared to RN46A-V1 and shams, which withdrew 4.01 ± 0.23 and 3.92 ± 0.18 times.

Similarly with 9.96-mN von Frey filaments, hemisection resulted in statistically significant ($P < 0.05$) increases in paw-withdrawal frequencies of $4.44 \pm$

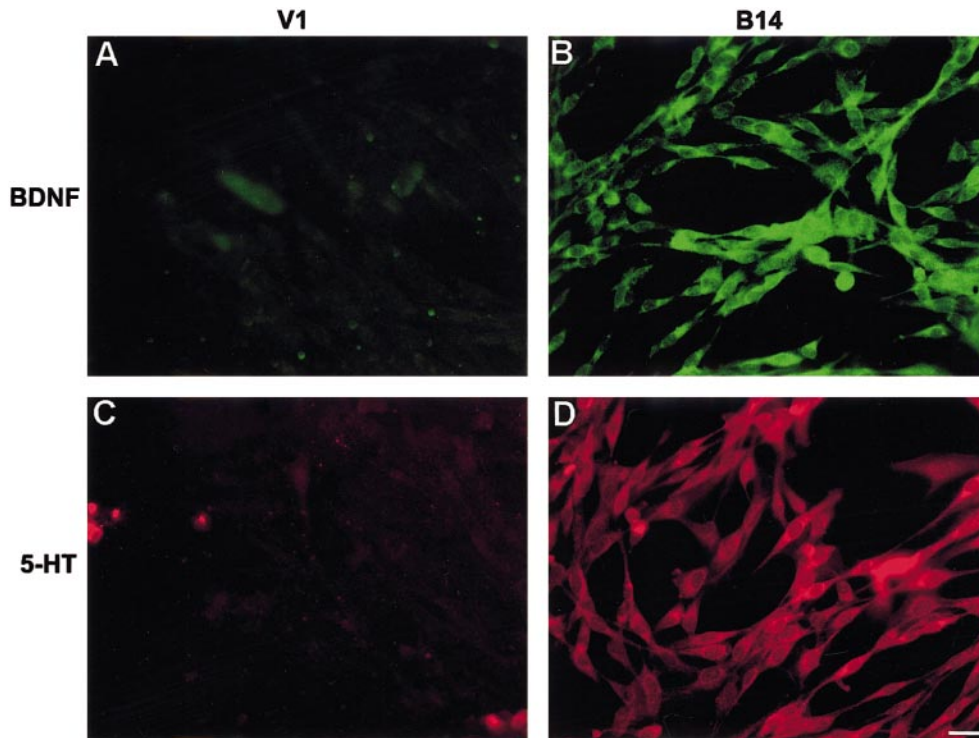


FIG. 4. *In vitro* BDNF and 5-HT immunofluorescence photomicrographs of confluent (3×10^6 cells) RN46A-B14 and RN46A-V1 cells proliferated for 3 days at 33°C in DMEM/F-12/10% FBS/G418/PS. BDNF staining is absent in RN46A-V1 (A) but present in RN46A-B14 (B) cells. 5-HT immunofluorescence is absent in RN46A-V1 (C) but demonstrated in RN46A-B14 cells (D). Neuronal morphology is evident in both populations. Scale bar, $150 \mu\text{m}$.

0.35, 4.37 ± 0.25 , and 4.40 ± 0.31 for RN46A-B14, RN46A-V1, and sham groups in forelimbs, from a normalized baseline of 1.85. Hindlimbs also developed mechanical allodynia, increasing from 0.45 to 4.39 ± 0.34 , 4.32 ± 0.20 , and 4.22 ± 0.22 in RN46A-B14, RN46A-V1, and sham groups, respectively. After receiving RN46A-B14, RN46A-V1, or no transplant, beginning at 36 days significant reductions in paw withdrawals were observed in animals that received RN46A-B14 cell transplants compared to RN46A-V1 and nontransplanted animals, and frequencies of paw withdrawal remained significantly decreased 28 days following transplantation. In forelimbs, RN46A-B14 animals withdrew 3.18 ± 0.32 times, while RN46A-V1 and sham animals withdrew 4.52 ± 0.22 and 4.18 ± 0.37 times, respectively. In hindlimbs, RN46A-B14 animals scored 2.95 ± 0.41 while RN46A-V1 and shams withdrew 5.27 ± 0.32 and 4.77 ± 0.41 times.

With a high-intensity 204.1-mN filament 28 days after hemisection, animals developed statistically significant ($P < 0.05$) increases in paw-withdrawal frequencies. In forelimbs, RN46A-B14, RN46A-V1, and no-transplant groups increased from 2.85 to 5.86 ± 0.31 , 6.90 ± 0.22 , and 5.97 ± 0.37 , respectively. Hindlimb frequency increased from 3.01 to 6.35 ± 0.56 , 6.49 ± 0.44 , and 6.47 ± 0.35 , for RN46A-B14, RN46A-V1, and no-transplant groups, respectively. After re-

ceiving RN46A-B14, RN46A-V1, or no transplant, beginning at 36 days significant reductions in paw withdrawals were observed in animals that received RN46A-B14 cell transplants compared to RN46A-V1 and nontransplanted animals, and frequencies of paw withdrawal remained significantly decreased 28 days following transplantation. In forelimbs at 4.79 mN strength RN46A-B14 animals withdrew 2.1 ± 0.18 times, while RN46A-V1 and sham animals withdrew their paws 3.91 ± 0.15 and 3.42 ± 0.20 times, respectively. Hindlimb withdrawal frequency in RN46A-B14 animals was 3.47 ± 0.58 while RN46A-V1 and shams withdrew 5.71 ± 0.64 and 5.68 ± 0.62 times.

For the pinprick test, responses were maximal at baseline (10 of 10), and hemisection did not alter the response to this stimulus (Fig. 7). RN46A-B14, RN46A-V1, or sham animals did not demonstrate statistically significant differences in pinprick response incidence at any time point or between surgical groups.

Thermal hyperalgesia. Spinal hemisection resulted in paw-withdrawal latencies accompanied by complex aversion behaviors that were significantly ($P < 0.05$) decreased compared to presurgical values for both fore- and hindlimbs (Figs. 8A and 8B). Pretransplant forelimb latencies in animals to receive RN46A-B14 or RN46A-V1 or no transplant were decreased from 12.5

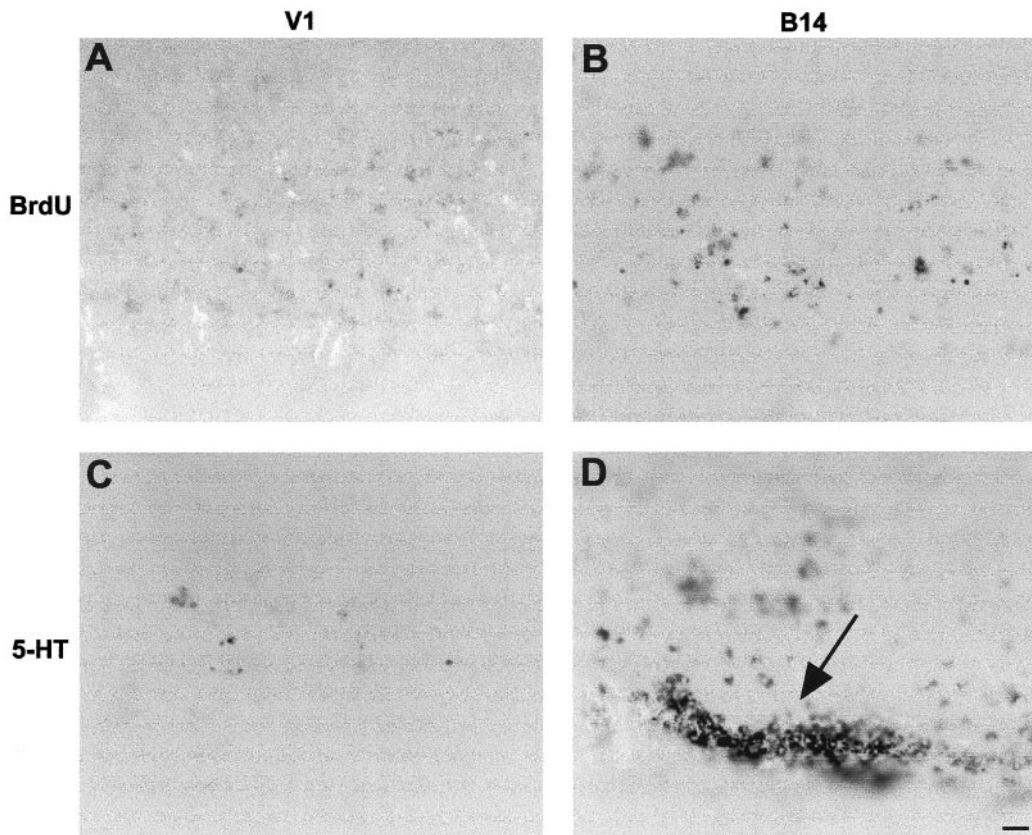


FIG. 5. *In vivo* photomicrographs of transplanted RN46A-B14 and RN46A-V1 cells taken 28 days after transplantation at spinal segments L1–L5. Both RN46A-V1 (A) and RN46A-B14 (B) cells stain for BrdU; however, cell clusters and more surviving cells are observed in the RN46A-B14 transplants. 5-HT immunoreactivity is observed only in RN46A-B14 (D) transplants and not in RN46A-V1 (C). In all specimens, cells remain clustered (arrow) at the original site of transplantation and do not migrate into host parenchyma or across the surface of the spinal cord. Scale bar, 50 μ m.

to 6.70 ± 0.36 , 6.55 ± 0.99 , and 5.65 ± 0.73 s, respectively. Hindlimb withdrawal latencies were similarly decreased, from 20.31 to 10.4 ± 1.35 , 9.08 ± 0.56 , and 11.46 ± 1.28 s in RN46A-B14, RN46A-V1, and non-transplant groups. Thus, limb withdrawals occurred at a shorter latency and consequently at a lower temperature, indicating development of thermal hyperalgesia.

After transplantation, only animals receiving RN46A-B14 cell transplants demonstrated significant ($P < 0.05$) increases in paw-withdrawal latency for both forelimbs and hindlimbs. For forelimbs at 28 days posttransplant, RN46A-B14 animals withdrew at 10.89 ± 0.86 s while RN46A-V1 and shams took 7.86 ± 1.44 and 8.13 ± 1.00 s, respectively. Hindlimbs also demonstrated increased latencies with RN46A-B14 animals at 20.53 ± 1.50 while RN46A-V1 and shams took 14.08 ± 1.20 and 12.96 ± 1.32 s, respectively. With the RN46A-B14 transplant, withdrawal latencies approximated preinjury levels, indicating a near-return to baseline paw-withdrawal responses.

Drug testing. Intrathecal injections of vehicle, methysergide, or fluvoxamine altered behavioral responses to von Frey mechanical and thermal stimuli 1

week following cell transplantation (Table 1). Intrathecal injection of the selective 5-HT antagonist methysergide significantly ($P < 0.05$) increased the responsiveness to mechanical and thermal stimuli in all groups and reversed the antinociceptive effects of RN46A-B14 cell transplants in both thermal and mechanical tests without compromising locomotor function assessed by the BBB scale or on normal pain sensation (pin). The magnitude of the effect of methysergide did not differ between groups. These results suggest that basal behavioral effects of endogenous 5-HT were eliminated as well as effects of exogenous 5-HT produced by RN46A-B14 transplants. In most but not all cases, methysergide affected both fore- and hindlimb behaviors.

Fluvoxamine, a highly selective serotonin reuptake inhibitor, significantly ($P < 0.05$) enhanced the effectiveness of RN46A-B14 transplants in both thermal and mechanical tests, without affecting normal locomotor function or responses to normally noxious stimuli (pin). Fluvoxamine also had an effect on sham and RN46A-V1 animals, but as with methysergide, effects were most pronounced in RN46A-B14 animals most

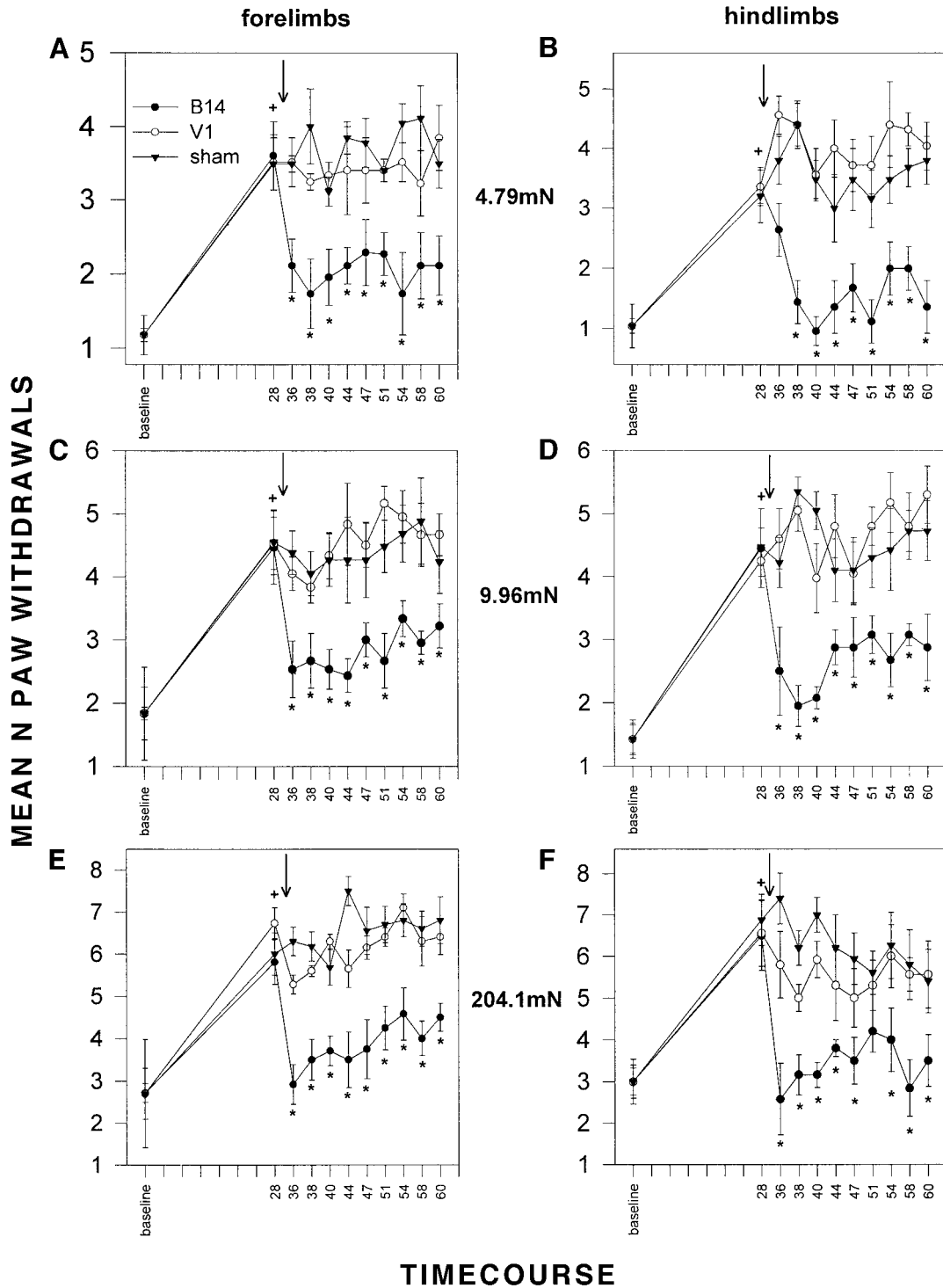


FIG. 6. Change in mean number of paw withdrawals (mean \pm SD) of combined forelimbs (A, C, E) and hindlimbs (B, D, F) plotted in days after T13 hemisection and lumbar transplantation (arrow) of RN46A-B14, RN46A-V1, or no cells, to various strengths of von Frey mechanical stimuli (milliNewton force generated) compared to baseline and various injury conditions. All groups demonstrated significant ($^+P < 0.05$) development of mechanical allodynia by 28 days after hemisection injury. Immediately after insertion, RN46A-B14 cells but not RN46A-V1 or sham transplants produced significantly ($*P < 0.05$) decreased withdrawal frequency compared to pretransplant conditions, which persisted for at least 28 days in both forelimbs and hindlimbs at low (A, B), medium (C, D), and high intensity (E, F) filament strengths.

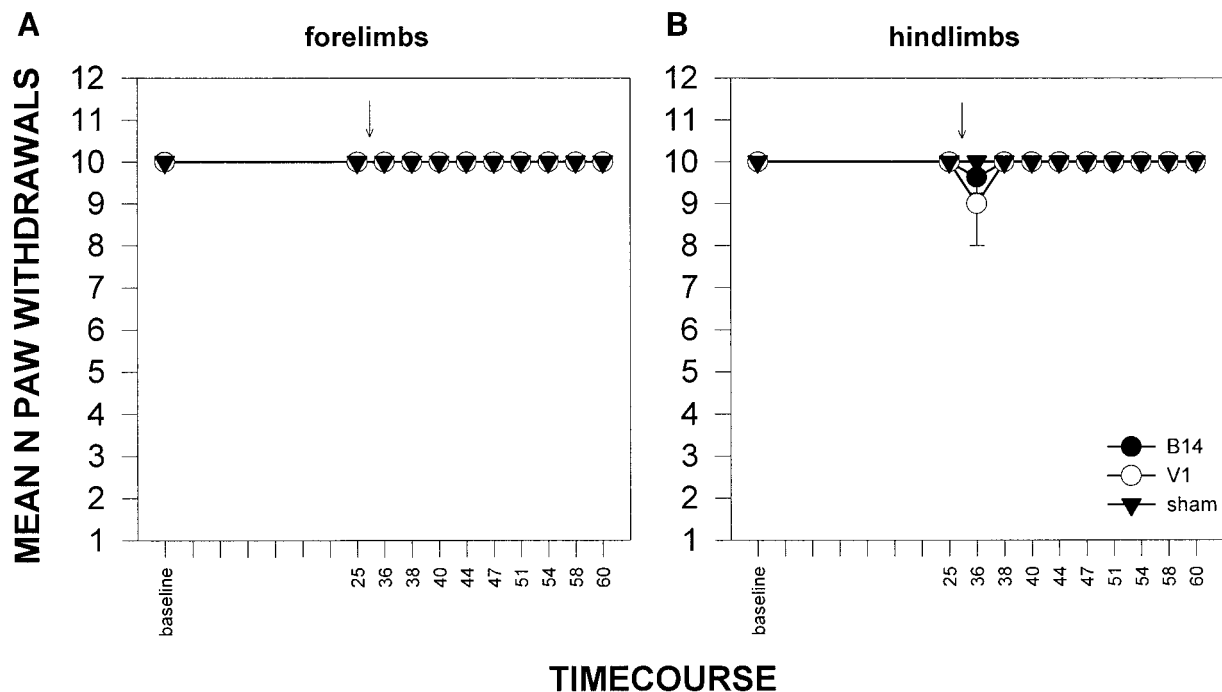


FIG. 7. Mean \pm SD number of paw withdrawals to noxious pin stimulus in forelimbs (A) and hindlimbs (B) following T13 hemisection injury and L1 transplantation (arrow) of RN46A-B14, RN46A-V1, or no cells. No animals in any group demonstrated significant analgesia from this normally noxious stimuli at any time point in both forelimbs and hindlimbs, before or after hemisection or transplantation, indicating preservation of responses to normally nocifensive stimuli.

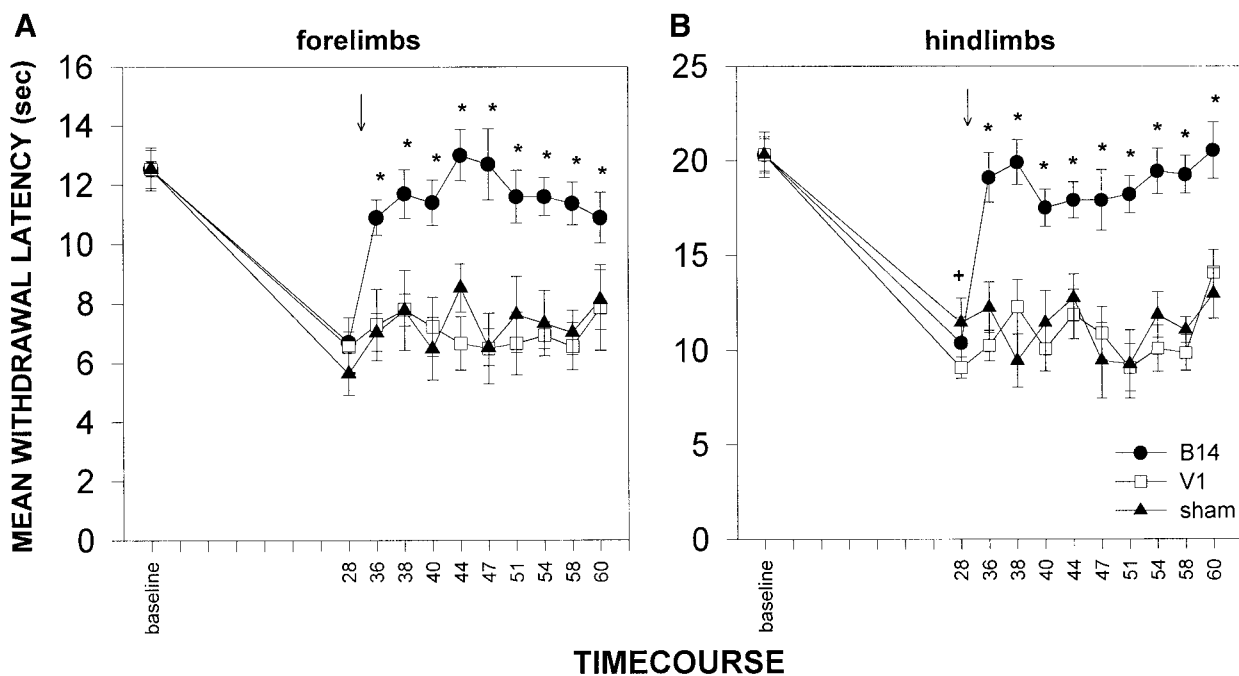


FIG. 8. Mean \pm SD changes in paw-withdrawal latency (seconds) to radiant thermal stimulus for forelimbs (A) and hindlimbs (B) following T13 hemisection injury and lumbar transplantation (arrow) of RN46A-B14, RN46A-V1, or no cells. All groups demonstrate significant ($*P < 0.05$) development of thermal hyperalgesia by 28 days after hemisection injury. Immediately following transplantation, only RN46A-B14-receiving animals demonstrated significant ($*P < 0.05$) increases in paw-withdrawal latency for both forelimbs and hindlimbs after transplantation, indicating decreases in thermal hyperalgesia, which persisted for at least 28 days.

TABLE 1

Group	BBB score		Thermal		4.79-mN von Frey		9.96-mN von Frey		204.1-mN von Frey		Pin	
	Hind	Fore	Hind	Fore	Hind	Fore	Hind	Fore	Hind	Fore	Hind	Fore
Sham+vehicle	14.2 ± 1.77	7.04 ± 0.64	11.2 ± 1.34	4.21 ± 0.34	3.52 ± 0.28	4.24 ± 0.30	4.19 ± 0.33	6.11 ± 0.28	6.13 ± 0.29	10.0 ± 0.00	10.0 ± 0.00	10.0 ± 0.00
Sham+methy	14.0 ± 1.47	5.88 ± 0.15†	5.66 ± 0.45†	4.99 ± 0.21*	5.23 ± 0.22*	5.85 ± 0.31*	5.91 ± 0.37*	6.68 ± 0.62	7.98 ± 1.21*	10.0 ± 0.00	10.0 ± 0.00	9.63 ± 0.38
Sham+fluvox	13.7 ± 0.63	11.7 ± 1.41*	13.2 ± 1.97	3.67 ± 0.22†	2.88 ± 0.34†	3.56 ± 0.28†	3.49 ± 0.29†	5.25 ± 0.96†	5.37 ± 1.03	10.0 ± 0.00	10.0 ± 0.00	10.0 ± 0.00
V+vehicle	13.4 ± 1.28	7.29 ± 1.20	10.2 ± 0.80	4.28 ± 0.31	3.49 ± 0.09	4.01 ± 0.27	4.65 ± 0.34	5.79 ± 0.87	4.47 ± 0.26	10.0 ± 0.00	10.0 ± 0.00	10.0 ± 0.00
V+methy	13.7 ± 0.63	5.01 ± 0.87†	5.2 ± 1.17†	5.21 ± 0.25*	4.98 ± 0.27*	5.18 ± 0.37*	5.65 ± 0.38*	6.82 ± 0.99*	5.25 ± 1.30*	10.0 ± 0.00	10.0 ± 0.00	10.0 ± 0.00
V+fluvox	14.2 ± 0.63	11.9 ± 1.83*	14.2 ± 3.51	3.37 ± 0.28†	3.15 ± 0.19†	3.30 ± 0.28†	4.13 ± 0.33	5.15 ± 0.98	3.01 ± 1.07†	10.0 ± 0.00	10.0 ± 0.00	10.0 ± 0.00
B14+vehicle	15.6 ± 0.87	13.6 ± 0.52	17.7 ± 1.44	2.02 ± 0.20	1.21 ± 0.20	2.65 ± 0.22	2.69 ± 0.32	3.25 ± 0.19	3.02 ± 0.24	10.0 ± 0.00	10.0 ± 0.00	10.0 ± 0.00
B14+methy	15.2 ± 0.75	8.20 ± 0.57†	10.2 ± 1.14†	4.00 ± 0.31*	1.72 ± 0.24*	3.27 ± 0.23*	3.50 ± 0.42*	4.48 ± 1.14*	4.13 ± 1.35*	10.0 ± 0.00	10.0 ± 0.00	10.0 ± 0.00
B14+fluvox	15.8 ± 0.73	18.0 ± 1.33*	21.7 ± 1.93*	1.25 ± 0.20†	0.68 ± 0.32†	2.03 ± 0.24†	2.12 ± 0.24†	2.82 ± 1.45†	2.01 ± 0.87	10.0 ± 0.00	10.0 ± 0.00	10.0 ± 0.00

Note. Effects of intrathecal vehicle (20 μ l), 5-HT antagonist (methysergide; 125 μ g/kg), or the selective 5-HT reuptake inhibitor (fluvoxamine; 75 μ g/kg) on mean \pm SD locomotor function (BBB), thermal withdrawal latencies (s), and frequency of paw withdrawals to mechanical stimulus 10 days after transplantation of RN46A-B14, RN46A-V1, or no cells after T13 spinal hemisection injury. In all groups, BBB scores were unaffected by i.t. drug, while significant ($P < 0.05$) modulation of 5-HT-mediated responses to mechanical and thermal stimuli were evident in both forelimbs and hindlimbs.

* $P < 0.05$ increase from vehicle.

† $P < 0.05$ decrease from vehicle.

likely due to higher concentrations of 5-HT from both endogenous and exogenous sources. At 90 min following injection of all drugs, there was no permanent desensitization, sensitization, or gross alterations in behavior as determined by postwashout behavioral tests, which showed no significant difference compared to predrug behavioral responses.

DISCUSSION

The present study confirms earlier work showing that following T13 spinal hemisection rats spontaneously recover considerable ipsilateral hindlimb locomotor function, but develop behavior consistent with abnormal nociceptive behaviors bilaterally in both forelimbs and hindlimbs (17). These effects are partially due to changes in serotonergic elements in the spinal cord (44). Here we report that transplantation of a conditionally immortalized 5-HT- and BDNF-secreting cell line survives for at least 30 days, without evidence of proliferation (with PCNA) or immune response (with ED-1), and secretes 5-HT and BDNF into the CSF. These results correspond with tissue analysis of RN46A transplantation after SCI in a parallel study from our laboratory (43). Behaviorally, RN46A-B14 cell grafts promote locomotor recovery and attenuate mechanical allodynia and thermal hyperalgesia without disrupting normal nociceptive behaviors for at least 28 days after spinal cord injury. Antinociceptive effects produced by exogenous 5-HT secreted by the transplants were demonstrated by i.t. injections of a 5-HT antagonist and a 5-HT uptake inhibitor, with no alteration in locomotor scores.

The modulation of spinal nociceptive transmission by serotonergic systems descending from the medullary raphe nuclei is well established (5). After spinal hemisection, interruption of serotonergic fibers results in alteration of both 5-HT and its transporter at acute and chronic time points in the lumbar enlargement on the ipsilateral side (44), and as such, interruption of descending serotonergic fibers by SCI confers release from inhibitory control onto dorsal horn nociceptive circuitry (94). After hemisection, spinal levels of 5-HT acutely diminish but return somewhat 4 weeks after injury, which is attributed to sprouting of contralateral fibers crossing the midline in laminae X to the ipsilateral side (83) and increased synthesis and metabolism in spared neuronal elements remaining after injury (41). The time course of reemergence of 5-HT after hemisection corresponds temporally with recovery of locomotion (83) and development of chronic central pain-like behavior (17), with the persistence of pain-related behaviors due in part to the inadequate return of 5-HT levels and changes in receptor and transporter population densities and activation states. Thus, alterations in 5-HT correlate with long-term, functional neurological outcome (31).

Work in our laboratory has shown that intrathecal 5-HT produces a significant modification of responses to mechanical and thermal stimuli in both forelimbs and hindlimbs after spinal hemisection—effects unaccompanied by any detectable changes in motor function, presumably due to low levels of 5-HT delivered intrathecally in that study (44). Pain modulation may be due to the ability of 5-HT to modify the effects of excitatory transmitters released by primary nociceptive afferents through the alteration of membrane excitability, or their postsynaptic influence, as 5-HT differentially modulates responses mediated by NMDA, quisqualate, and kainate (91). Additionally, wide dynamic range neurons made hyperexcitable after SCI (18) possess 5-HT receptors such as 5-HT_{1A} and 5-HT₃ (57, 75), which are thus subject to 5-HT modulation (100). In the present study we do not show complete reduction in pain-related behaviors after RN46A-B14 transplant—undoubtedly due to the presence of additional nociceptive modulators not examined in the present study that act in a combinatorial fashion.

It is also well known that 5-HT plays a role in locomotor function. Local spinal circuitry responsible for rhythmic control of limb movements, known as central pattern generators, is modulated by supraspinal descending serotonergic inputs (81). Following spinal transection, transplantation of embryonic raphe cells results in recovery of rhythmic locomotor function and increased responsiveness to reflex tests (92), only through transplantation at segments L1–L2 (77). In both reports, similar to our data, locomotor activity was not immediately restored but became evident only after several days. The effectiveness of 5-HT-secreting cells is hypothesized to be on the excitability of host neurons through increases in amplitude of monosynaptic reflexes in central pattern generator circuitry (98). Studies confirm that agonists for 5-HT directly depolarize α motor neurons (8) and that 5-HT can indirectly influence motoneuron excitability by attenuation of both excitatory and inhibitory synaptic inputs (58, 106).

RN46A-B14 transplants also produce BDNF, which is required for graft survival; however, its precise role here is difficult to elucidate. Neurotrophic factors such as NGF, BDNF, and NT-3 play a significant role in the development and perhaps maintenance of sensory neurons responsive to temperature and tactile pain (52, 62), as both motoneurons and sensory neurons express trkB (54, 63, 85). BDNF is localized in terminals of primary sensory neurons (3), which colocalize with CGRP-containing arborizations of afferent nociceptive C-fiber terminals (67, 108). As such, it could be released with activation of afferents (38) and modulate sensory stimuli. In a peripheral inflammation model, endogenous BDNF may play a different role in central sensitization, with increased NMDA receptor-mediated excitability (95) and induction of c-fos (50) after

exogenous BDNF application. This is in contrast to nerve injury, in which BDNF is able to reduce GABA_A-mediated conductances (73), suggesting that BDNF may facilitate nociceptive input from those terminals. Serotonin transporter function has been shown to be modulated by BDNF in B lymphoblast cell lines (69), which could increase extracellular 5-HT through inhibition of uptake. Additionally, BDNF has been proposed to modulate 5-HT levels through increasing steady-state tryptophan hydroxylase mRNA levels (90). While some report that BDNF may potentiate spinal nociceptive reflex responses in the spinal dorsal horn (82), and that it contributes to hypersensitivity to low-intensity tactile stimulation after peripheral inflammation (62), these data are inconsistent with our findings using a chronic model of central pain.

The role of BDNF in spinal cord injury is becoming better known. Continuous infusion into the injured cord reduces the necrotic zone and supports neuronal survival (72) since BDNF possesses neurotrophic functions in addition to its role as a target-derived neurotrophic factor (2, 53). After spinal injury, trkB mRNA increases correlate with axonal sprouting (34) in rubrospinal and bulbo-spinal pathways (59, 105) and in corticospinal tracts (CST) (35). Because virtually all CST neurons express trkB mRNA, whereas only half express BDNF, some CST fibers must receive BDNF support through a paracrine mechanism (36). Behaviorally, chronic infusion of BDNF stimulates hindlimb activity after contusion and is associated with enhanced growth of cholinergic fibers (47). Transplantation of BDNF-secreting fibroblasts following contusion injury results in improved locomotor recovery (51).

Since behavioral effects of the transplant are immediately evident, it is hypothesized that both 5-HT and BDNF secreted by the RN46A-B14 cells contribute to anti-nociception and that secretagogues are acting on *in situ* elements. This type of effect is analogous to i.t.-delivered 5-HT-acting agents after hemisection (44). In this case, antagonists or reuptake inhibitors acutely modify pain-related behaviors, suggesting restoration of inhibitory tone through receptor-mediated changes in membrane polarization and excitability. With regard to locomotor function, it seems that the effectiveness of the RN46A-B14 cells is cumulative, such that locomotor recovery seems to improve over time in the RN46A-B14-receiving animals. It may be that BDNF induces sprouting of locomotor circuitry, which affects output to locomotor behavior only after a period of 1–2 weeks after transplantation. That BDNF and not 5-HT, in our case, is playing an important locomotor role is further evidenced by a lack of alteration in locomotor function with i.t. 5-HT-acting agents. This hypothesis could be tested by administration of BDNF antagonists; however, the BDNF requirement of the RN46A-B14 cell line for 5-HT secretion and survival renders this approach as unfeasible.

Considering what is classically taught about pain pathways, it may seem perplexing that forelimb and bilateral sensory responses are altered after hemisection. In patients, the Brown-Sequard syndrome (spinal hemisection) is characterized by ipsilateral hemiparalysis and contralateral hypalgesia (10), but can also result in pain bilaterally below the lesion (40, 78) due to changes in contralaterally projecting primary nociceptive afferents (9, 24). In addition, pain fibers terminate not only at and below the level of innervation but also above (24, 56, 96). There are anatomical substrates in place for supraspinal transmission of peripherally evoked nociceptive information caudal to the lesion, both ipsilaterally and contralaterally in humans (60, 79) and in rats (97). The contralaterally affected area following unilateral spinothalamic (STT) tractotomy can be subserved by the uninterrupted spinocervicothalamic tract, which carries nociceptive information (28, 102), and ipsilaterally projecting (18%) STT cells (11, 76). Moreover, the spinoreticular tract mediates somatosensation bilaterally (80) and short-fiber multisegmental propriospinal pathways are able to relay similar information from one side of the cord to another (4, 28, 87). Indirect evidence exists that pain and temperature information are also transmitted rostrally by the ipsilateral dorsal column since primary afferent C fibers are found within the dorsal funiculus (74). Thus, several neural circuits exist which can account for the supraspinal nociceptive information outside of the conventionally accepted STT pathways after SCI, and as such, alterations in sensory processing are not necessarily dependent upon the sidedness of SCI, but on the tracts and cellular processes altered.

With regard to the remarkable development of pain in the forelimbs, there is evidence of neural circuitry which would permit their involvement after thoracic hemisection. In quadrupedal animals it is well documented that locomotor circuits such as flexor/crossed extensor reflex exist (39, 89). In this reflex, noxious stimulation to one hindlimb elicits an effect in that limb as well as the contralateral forelimb. For this study, it is important to note that 5-HT has been implicated in this and other nociceptive reflexes such as tail-flick (76) and monosynaptic withdrawal reflex (39, 89), in both facilitory and inhibitory manners (13). Also, pathways involved in locomotion employ reciprocal side-to-side propriospinal (23), and intersegmental connections (20–22) have been demonstrated in nociceptive afferent fibers through collateral pathways (48, 68). In addition, long cervical–lumbar (1) and complementary lumbrosacro-cervical connections exist (26) which directly synapse onto interneurons involved in nociceptive and cutaneous reflexive pathways of hind- and forelimbs (91). In cat, and presumably other mammals, there are numerous individual nociceptive STT cells clustered in the first few cervical spinal segments which respond to peripheral noxious stimulation from

the whole body (70, 71, 93). These pathways are possible substrates for transmission of hindlimb somatosensation to cervical segments and supraspinally.

As such, it is hypothesized that altered lesion circuitry in thoracic or lumbar segments can affect both forelimb and hindlimb behavioral reflexes through disruption and modification of these pathways throughout the entire neuraxis. Using this reasoning, a unilateral segmental disruption of the circuit would alter the pathophysiology of the remaining neural circuitry. Consequently, i.t.-delivered agents, with limited diffusion capabilities (44, 66, 86), and transplantation of cells at or near the hemisection site are predicted to be effective in modulating forelimb and hindlimb sensitivity, by restoring 5-HT levels in local neural circuitry and affecting neural inputs that are many segments rostral and caudal, which is what the present study demonstrates.

In summary, our results support the therapeutic potential of subdurally transplanted 5-HT- and BDNF-secreting cells in the treatment of locomotor dysfunction and chronic central pain following spinal injury. Cell transplantation approaches have met with success in the treatment of clinical neuropathic pain in cancer patients (55) and should be considered in pragmatic designs of clinical treatment strategies for pain syndromes after SCI.

ACKNOWLEDGMENTS

The authors thank Mr. Cannon Clifton for his technical assistance, Ms. Debbie Pavlu for her administrative assistance, Mr. Tomas Lopez for his guidance and friendship, Dr. Gregg T. Nagle for the use of cell culture facilities, and Dr. Scott R. Whittemore for use of the RN46A cell line. This work was supported by the Spinal Cord Research Foundation of the Paralyzed Veterans of America, the Kent Waldrep National Paralysis Foundation, Mission Connect of TIRR, and NIH Grants NS11255 and NS39161.

REFERENCES

1. Adamson, J., R. A. Zappulla, A. Fraser, J. Ryder, and L. I. Malis. 1989. Effects of selective spinal cord lesions on the spinal motor evoked potential (MEP) in the rat. *Electroencephalogr. Clin. Neurophysiol.* **74**: 469–480.
2. Altar, C. A., J. A. Siuciak, P. Wright, N. Y. Ip, R. M. Lindsay, and S. J. Wiegand. 1994. In situ hybridization of trkB and trkC receptor mRNA in rat forebrain and association with high-affinity binding of [¹²⁵I]BDNF, [¹²⁵I]NT-4/5 and [¹²⁵I]NT-3. *Eur. J. Neurosci.* **6**: 1389–1405.
3. Apfel, S. C., D. E. Wright, A. M. Wiideman, C. Dormia, W. D. Snider, and J. A. Kessler. 1996. Nerve growth factor regulates the expression of brain-derived neurotrophic factor mRNA in the peripheral nervous system. *Mol. Cell. Neurosci.* **7**: 134–142 (doi:10.1006/mcne.1996.0010).
4. Basbaum, A. I. 1973. Conduction of the effects of noxious stimulation by short-fiber multisynaptic systems of the spinal cord in rat. *Exp. Neurol.* **40**: 699–716.
5. Basbaum, A. I., and H. L. Fields. 1984. Endogenous pain systems: Brainstem pathways and endorphin circuitry. *Annu. Rev. Neurosci.* **7**: 309–338.

6. Basso, M., M. S. Beattie, and J. C. Bresnahan. 1995. A sensitive and reliable locomotor rating scale for open field testing in rats. *J. Neurotrauma* **12**: 1–21.
7. Bennet, G. J., and Y. K. Xie. 1988. A peripheral motoneuropathy in rat that produces disorders of pain sensation like those seen in man. *Pain* **33**: 87–107.
8. Berger, A. J., D. A. Bayliss, and F. Viana. 1992. Modulation of neonatal rat hypoglossal motoneuron excitability by serotonin. *Neurosci. Lett.* **143**: 164–168.
9. Bowsher, D. 1988. Contralateral mirror-image pain following anterolateral cordotomy. *Pain* **33**: 63–65.
10. Brown-Sequard, C. D. 1868. Lectures on the physiology and pathology of the central nervous system and on the treatment of organic nervous affections. *Lancet* 2593–2596.
11. Burstein, R., R. J. Dado, and G. J. Giesler. 1990. The cells of origin of the spinothalamic tract of the rat: A quantitative reexamination. *Brain Res.* **511**: 329–337.
12. Cairnes, D. M., R. H. Adkins, and M. D. Schorr. 1996. Pain and depression in acute traumatic spinal cord injury: Origins of chronic problematic pain? *Arch. Phys. Med. Rehab.* **77**: 329–335.
13. Calejesan, A. A., M. H. Chang, and M. Zhuo. 1998. Spinal serotonergic receptors mediate facilitation of a nociceptive reflex by subcutaneous formalin injection into the hindpaw in rats. *Brain Res.* **798**: 46–54.
14. Cejas, P. J., M. Martinez, S. Karmally, M. McKillop, J. McKillop, J. A. Plunkett, M. Oudega, and M. J. Eaton. 2000. Lumbar transplant of neurons genetically modified to secrete brain-derived neurotrophic factor attenuates allodynia and hyperalgesia after sciatic nerve constriction. *Pain* **86**: 195–210.
15. Celada, P., J. A. Siuciak, T. M. Tran, C. A. Altar, and J. M. Tepper. 1996. Local infusion of brain-derived neurotrophic factor modifies the firing pattern of dorsal raphe serotonergic neurons. *Brain Res.* **712**: 293–298.
16. Choi, Y., Y. W. Yoon, H. S. Na, S. H. Kim, and J. M. Chung. 1994. Behavioral signs of ongoing pain and cold allodynia in a rat model of neuropathic pain. *Pain* **59**: 369–376.
17. Christensen, M. D., A. W. Everhart, J. T. Pickleman, and C. E. Hulsebosch. 1996. Mechanical and thermal allodynia in chronic central pain following spinal cord injury. *Pain* **68**: 97–107.
18. Christensen, M. D., and C. E. Hulsebosch. 1997. Chronic central pain after spinal cord injury. *J. Neurotrauma* **14**: 517–537.
19. Cirulli, F., A. Berry, and E. Alleva. 2000. Intracerebroventricular administration of brain-derived neurotrophic factor in adult rats affects analgesia and spontaneous behavior but not memory retention in a Morris water maze task. *Neurosci. Lett.* **287**: 207–210.
20. Clarke, R. W., J. Harris, and A. K. Houghton. 1996. Spinal 5-HT-receptors and tonic modulation of transmission through a withdrawal reflex pathway in the decerebrated rabbit. *Br. J. Pharmacol.* **119**: 1167–1176.
21. Clarke, R. W., J. Ogilvie, and A. K. Houghton. 1997. Enhancement and depression of spinal reflexes by 8-hydroxy-2-(di-*n*-propylamino)tetralin in the decerebrated and spinalized rabbit: Involvement of 5-HT_{1A}- and non-5-HT_{1A}-receptors. *Br. J. Pharmacol.* **122**: 631–638.
22. Cowley, K. C., and B. J. Schmidt. 1997. Regional distribution of the locomotor pattern-generating network in the neonatal rat spinal cord. *J. Neurophysiol.* **77**: 247–259.
23. Crick, H., N. A. Manuel, and D. I. Wallis. 1994. A novel 5-HT receptor or a combination of 5-HT receptor subtypes may mediate depression of a spinal monosynaptic reflex in vitro. *Neuropharmacology* **33**: 897–904.
24. Culbertson, J. L., D. E. Hines, D. L. Kimmel, and P. B. Brown. 1979. Contralateral projection of primary afferent fibers to mammalian spinal cord. *Exp. Neurol.* **64**: 83–97.
25. Deng, Y. S., J. H. Zhong, and X. F. Zhou. 1999. BDNF is involved in sympathetic sprouting in the dorsal root ganglia following peripheral nerve injury in rats. *Neurotoxicol. Res.* **1**: 311–322.
26. Diener, P. S., and B. S. Bregman. 1998. Fetal spinal cord transplants support the development of target reaching and coordinated postural adjustments after neonatal cervical spinal cord injury. *J. Neurosci.* **18**: 763–778.
27. Dirig, D. M., A. Salami, M. L. Rathbun, G. T. Ozaki, and T. L. Yaksh. 1997. Characterization of variables defining hindpaw withdrawal latency evoked by radiant thermal stimuli. *J. Neurosci. Methods* **76**: 183–191.
28. Downie, J. W., D. G. Ferrington, L. S. Sorkin, and W. D. Willis. 1988. The primate spinocervicothalamic pathway: Responses of cells of the lateral cervical nucleus and spinocervical tract to innocuous and noxious stimuli. *J. Neurophysiol.* **59**: 861–885.
29. Eaton, M. J., D. L. Santiago, H. A. Dancausse, and S. R. Whittemore. 1997. Lumbar transplants of immortalized serotonergic neurons alleviate chronic neuropathic pain. *Pain* **72**: 59–69.
30. Emfors, P., C. Wetmore, L. Olson, and H. Persson. 1990. Identification of cells in rat brain and peripheral tissues expressing mRNA for members of the nerve growth factor family. *Neuron* **5**: 511–526.
31. Faden, A. I., A. Gannon, and A. I. Basbaum. 1988. Use of serotonin immunocytochemistry as a marker of injury severity after experimental spinal trauma in rats. *Brain Res.* **450**: 94–100.
32. Feraboli-Lohnherr, D., D. Orsal, A. Yakovlev, M. Gimenez y Ribotta, and A. Privat. 1997. Recovery of locomotor activity in the adult chronic spinal rat after sublesional transplantation of embryonic nervous cells: Specific role of serotonergic neurons. *Exp. Brain Res.* **113**: 443–454.
33. Foster, G. A., M. H. Roberts, L. S. Wilkinson, A. Bjorklund, F. H. Gage, T. Hokfelt, M. Schultzberg, and T. Sharp. 1989. Structural and functional analysis of raphe neurone implants into denervated rat spinal cord. *Brain Res. Bull.* **22**: 131–137.
34. Frisen, J., V. M. K. Verge, S. Cullheim, H. Persson, K. Fried, D. S. Middlemas, T. Hunter, T. Hokfelt, and M. Risling. 1992. Increased levels of trkB mRNA and trkB protein-like immunoreactivity in the injured rat and cat spinal cord. *Proc. Natl. Acad. Sci. USA* **89**: 11282–11286.
35. Giehl, K. M., and W. Tetzlaff. 1996. BDNF and NT-3, but not NGF, prevent axotomy-induced death of rat corticospinal neurons in vivo. *Eur. J. Neurosci.* **8**: 1167–1175.
36. Giehl, K. M., A. Schutte, P. Mestres, and Q. Yah. 1998. The survival-promoting effect of glial cell line-derived neurotrophic factor on axotomized corticospinal neurons in vivo is mediated by an endogenous brain-derived neurotrophic factor mechanism. *J. Neurosci.* **18**: 7351–7360.
37. Gimenez y Ribotta, M., M. Rajaofetra, C. Morin-Richaud, G. Alonso, D. Bochelen, F. Sandillon, A. Legrand, M. Mersel, and A. Privat. 1995. Oxysterol (7 beta-hydroxycholesteryl-3-oleate) promotes serotonergic reinnervation in the lesioned rat spinal cord by reducing glial reaction. *J. Neurosci. Res.* **41**: 79–95.
38. Griesbeck, O., M. Canossa, G. Campana, A. Gartner, M. C. Hoener, H. Nawa, R. Kolbeck, and H. Thoenen. 1999. Are there differences between the secretion characteristics of NGF and BDNF? Implications for the modulatory role of neurotrophins in activity-dependent neuronal plasticity. *Microsc. Res. Tech.* **45**: 262–275.
39. Grillner, S. 1975. Locomotion in vertebrates: Central mechanisms and reflex interaction. *Physiol. Rev.* **55**: 247–304.
40. Guillian, G., and R. Garcia. 1931. Le syndrome de brown-sequard d'origine traumatique. *Ann. Med.* **29**: 361–385.
41. Hadjiconstantinou, M., P. Panula, Z. Lackovic, and N. H. Neff. 1984. Spinal cord serotonin: A biochemical and immunohistochemical study following transection. *Brain Res.* **26**: 245–254.

42. Hains, B. C., K. M. Chastain, A. W. Everhart, and C. E. Hulsebosch. 2000. Transplants of adrenal medullary chromaffin cells reduce forelimb and hindlimb allodynia in a rodent model of chronic central pain after spinal cord hemisection injury. *Exp. Neurol.* **164**: 426–437 (doi:10.1006/exnr.2000.7439).
43. Hains, B. C., S. D. Fullwood, M. J. Eaton, and C. E. Hulsebosch. 2001. Subdural engraftment of serotonergic neurons following spinal hemisection restores spinal serotonin, down-regulates serotonin transporter, and increases BDNF tissue content in rat. *Brain Res.*, in press.
44. Hains, B. C., A. W. Everhart, and C. E. Hulsebosch. 2001. Serotonin and serotonin transporter mediated behavioral deficits after spinal hemisection are modulated by intrathecal 5-HT, antagonists, and reuptake inhibitors. Submitted for publication.
45. Hargreaves, K., R. Dubner, F. Brown, C. Flores, and J. Joris. 1988. A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain* **32**: 77–88.
46. Hofer, M. M., and Y. A. Barde. 1988. Brain-derived neurotrophic factor prevents neuronal death in vivo. *Nature* **331**: 261–262.
47. Jakeman, L. B., P. Wei, Z. Guan, and B. T. Stokes. 1988. Brain-derived neurotrophic factor stimulates hindlimb stepping and sprouting of cholinergic fibers after spinal cord injury. *Exp. Neurol.* **154**: 170–184.
48. Jankowska, E., A. Lundberg, and D. Stuart. 1983. Propriospinal control of interneurons in spinal reflex pathways from tendon organs in the cat. *Brain Res.* **261**: 317–320.
49. Johnson, R. A., A. J. Okragly, M. Haak-Frendscho, and G. S. Mitchell. 2000. Cervical dorsal rhizotomy increases brain-derived neurotrophic factor and NT-3 expression in the ventral spinal cord. *J. Neurosci.* **20**: 1–5.
50. Kerr, B. J., E. J. Bradbury, D. L. H. Bennett, P. M. Trivedi, P. Dassan, J. French, D. B. Shelton, S. B. McMahon, and W. E. N. Thompson. 1999. Brain-derived neurotrophic factor modulates nociceptive sensory inputs and NMDA-evoked responses in the rat spinal cord. *J. Neurosci.* **19**: 6138–6148.
51. Kim, D. H., P. H. Gutin, L. J. Noble, D. Nathan, J. S. Yu, and R. P. Nockels. 1996. Treatment with genetically engineered fibroblasts producing NGF or BDNF can accelerate recovery from traumatic spinal cord injury in the adult rat. *NeuroReport* **7**: 2221–2225.
52. Klein, R. 1994. Role of neurotrophins in mouse neuronal development. *FASEB J.* **8**: 738–744.
53. Kokaia, Z., M. Metsis, M. Kokaia, E. Elmer, and O. Lindvall. 1995. Co-expression of TrkB and TrkC receptors in CNS neurons suggests regulation by multiple neurotrophins. *NeuroReport* **6**: 769–772.
54. Koliatsos, V. E., R. E. Clatterbuck, J. W. Winslow, M. H. Cayouette, and D. L. Price. 1993. Evidence that brain-derived neurotrophic factor is a trophic factor for motor neurons in vivo. *Neuron* **10**: 359–367.
55. Lazorthes, Y., J. Sagen, B. Sallerin, J. Tkaczuk, H. Duplan, J. C. Sol, M. Tafani, and J. C. Bes. 2000. Human chromaffin cell graft into the CSF for cancer pain management: A prospective phase II clinical study. *Pain* **87**: 19–32.
56. Light, A. R., and E. R. Perl. 1979. Spinal termination of functionally identified primary afferent neurons with slowly conducting myelinated fibers. *J. Comp. Neurol.* **186**: 133–150.
57. Lin, Q., Y. B. Peng, and W. D. Willis. 1996. Antinociception and inhibition from the periaqueductal gray are mediated in part by spinal 5-hydroxytryptamine_{1A} receptors. *J. Pharm. Exp. Ther.* **276**: 958–967.
58. Lindsay, A. D., and J. L. Feldman. 1993. Modulation of respiratory activity of neonatal rat phrenic motoneurons by serotonin. *J. Physiol.* **461**: 213–233.
59. Liu, Y., D. Kim, B. T. Himes, S. Y. Chow, T. Schallert, M. Murray, A. Tessler, and I. Fischer. 1999. Transplants of fibroblasts genetically modified to express BDNF promote regeneration of adult rat rubrospinal axons and recovery of forelimb function. *J. Neurosci.* **19**: 4370–4387.
60. Loeser, J. D., A. A. Ward, and L. E. White. 1996. Chronic deafferentation of human spinal cord neurons. *J. Neurosurg.* **29**: 48–50.
61. Mamounas, L. A., M. E. Blue, J. A. Siuciak, and C. A. Altar. 1995. Brain-derived neurotrophic factor promotes the survival and sprouting of serotonergic axons in rat brain. *J. Neurosci.* **15**: 7929–7939.
62. Mannion, R. J., M. Costigan, I. Decosterd, F. Amaya, F., Q. P. Ma, J. C. Holstege, R. R. Ji, A. Acheson, R. M. Lindsay, G. A. Wilkinson, and C. J. Woolf. 1999. Neurotrophins: Peripherally and centrally acting modulators of tactile stimulus-induced inflammatory pain hypersensitivity. *Proc. Natl. Acad. Sci. USA* **96**: 9385–9390.
63. McMahon, S. B., M. P. Armanini, L. H. Ling, and H. S. Phillips. 1994. Expression and coexpression of Trk receptors in subpopulations of adult primary sensory neurons projecting to identified peripheral targets. *Neuron* **12**: 1161–1171.
64. Menei, P., C. Montero-Menei, S. R. Whittmore, R. P. Bunge, and M. B. Bunge. 1998. Schwann cells genetically modified to secrete human BDNF promote enhanced axonal regrowth across transected adult rat spinal cord. *Eur. J. Neurosci.* **10**: 607–621.
65. Mercadante, M. S. 1999. Problems of long-term spinal opioid treatment in advanced cancer pain. *Pain* **79**: 1–13.
66. Mestre, C., T. Pelissier, J. Fialip, G. Wilcox, and A. Eschaliere. 1994. A method to perform direct transcutaneous intrathecal injection in rats. *J. Pharm. Tox. Meth.* **32**: 197–200.
67. Michael, G. J., S. Averill, A. Nitkunan, M. Rattray, D. L. Bennett, Q. Yan, and J. V. Priestley. 1997. Nerve growth factor treatment increases brain-derived neurotrophic factor selectively in TrkA-expressing dorsal root ganglion cells and in their central terminations within the spinal cord. *J. Neurosci.* **17**: 8476–8490.
68. Miller, S., J. Van der Burg, and F. Van der Meche. 1975. Coordination of movements of the hindlimbs and forelimbs in different forms of locomotion in normal and decerebrate cats. *Brain Res.* **91**: 217–237.
69. Mossner, R., S. Daniel, D. Albert, A. Heils, O. Okladnova, A. Schmitt, and K. P. Lesch. 2000. Serotonin transporter function is modulated by brain-derived neurotrophic factor (BDNF) but not nerve growth factor (NGF). *Neurochem. Int.* **36**: 197–202.
70. Mrowczynski, W. 1997. Lamina IV–VI neurons of the second sacral segment projecting to the sixth cervical segment of the cat spinal cord. *Acta Neurobiol. Exp.* **57**: 189–195.
71. Nathan, P. W., and M. C. Smith. 1956. Some tracts of the anterior and lateral columns of the spinal cord. Pages 47–57 in R. S. Knighton and P. R. Dumke, Eds., *Pain*. Little, Brown, Boston.
72. Novikova, L., L. Novikov, and J. O. Kellerth. 1996. Brain-derived neurotrophic factor reduces necrotic zone and supports neuronal survival after spinal cord hemisection in adult rats. *Neurosci. Lett.* **220**: 203–206.
73. Oyelese, A. A., M. A. Rizzo, S. G. Waxman, and J. D. Kocsis. 1997. Differential effects of NGF and BDNF on axotomy-induced changes in GABA(A)-receptor-mediated conductance and sodium currents in cutaneous afferent neurons. *J. Neurophysiol.* **78**: 31–42.

74. Patterson, J. T., K. Chung, and R. E. Coggeshall. 1992. Further evidence for the existence of long ascending unmyelinated primary afferent fibers within the dorsal funiculus: Effects of capsaicin. *Pain* **49**: 117–120.
75. Peng, Y. B., Q. Lin, and W. D. Willis. 1996. The role of 5-HT₃ receptors in periaqueductal gray-induced inhibition of nociceptive dorsal horn neurons in rats. *J. Pharm. Exp. Ther.* **276**: 116–124.
76. Pitcher, G. M., K. Yashpal, T. J. Coderre, and J. L. Henry. 1995. Mechanisms underlying antinociception produced by heterosegmental noxious stimulation in the rat tail-flick test. *Neuroscience* **65**: 273–281.
77. Ribotta, M. G., J. Provencher, D. Feraboli-Lohnherr, S. Rossignol, A. Privat, A., and D. Orsal. 2000. Activation of locomotion in adult chronic spinal rats is achieved by transplantation of embryonic raphe cells reinnervating a precise lumbar level. *J. Neurosci.* **20**: 5144–5152.
78. Riddoch, G. 1938. The clinical features of central pain. *Lancet* **234**: 1093–1098, 1150–1156, 1205–1209.
79. Rintala, D. H., P. G. Loubser, J. Castro, K. A. Hart, and M. J. Fuhrer. 1998. Chronic pain in a community-based sample of men with spinal cord injury: Prevalence, severity, and relationship with impairment, disability, handicap, and subjective well-being. *Arch. Phys. Med. Rehab.* **79**: 604–614.
80. Roberts, W. A., and J. Wells. 1980. Extensive dual innervation and mutual inhibition by forelimb and hindlimb inputs to ventroposterolateral nucleus projection neurons in the rat. *Somatosens. Mot. Res.* **7**: 85–95.
81. Rossignol, S., J. P. Lund, and T. Drew. 1988. The role of sensory inputs in regulating patterns of rhythmical movements in higher vertebrates. A comparison between locomotion, respiration and mastication. Pages 201–283 in A. H. Cohen, S. Rossignol, and S. Grillner, Eds., *Neural Control of Rhythmic Movements in Vertebrates*. Wiley, New York.
82. Rutherford, L. C., S. B. Nelson, and G. G. Turrigiano. 1998. BDNF has opposite effects on the quantal amplitude of pyramidal neuron and interneuron excitatory synapses. *Neuron* **21**: 521–530.
83. Saruhashi, Y., W. Young, and R. Perkins. 1996. The recovery of 5-HT immunoreactivity in lumbosacral spinal cord and locomotor function after thoracic hemisection. *Exp. Neurol.* **139**: 203–213 (doi:10.1006/exnr.1996.0094).
84. Schabitz, W. R., S. Schwab, M. Spranger, and W. Hacke. 1997. Intraventricular brain-derived neurotrophic factor reduces infarct size after focal ischemia in rats. *J. Cereb. Blood Flow Metab.* **17**: 500–506.
85. Schecterson, L. C., and M. Bothwell. 1992. Novel roles for neurotrophins are suggested by BDNF and NT-3 mRNA expression in developing neurons. *Neuron* **9**: 449–463.
86. Schmauss, C., D. L. Hammond, J. W. Ochi, and T. L. Yaksh. 1983. Pharmacological antagonism of the antinociceptive effects of serotonin in the rat spinal cord. *Eur. J. Pharm.* **90**: 349–357.
87. Schoenen, J., and G. Grant. 1990. Spinal cord: Connections. Pages 77–92 in G. Paxinos, Ed., *The Human Nervous System*. Academic Press, New York.
88. Segatore, M. 1994. Understanding chronic pain after spinal cord injury. *J. Neurosci. Nurs.* **26**: 230–236.
89. Sherrington, C. S. 1910. Flexion–reflex of the limb, crossed extension–reflex, and reflex stepping and standing. *J. Physiol.* **40**: 28–121.
90. Siuciak, J. A., C. A. Altar, S. J. Wiegand, and R. M. Lindsay. 1994. Antinociceptive effect of brain-derived neurotrophic factor and neurotrophin-3. *Brain Res.* **633**: 326–330.
91. Skinner, R. D., R. J. Adams, and R. S. Rempel. 1980. Responses of long descending propriospinal neurons to natural and electrical types of stimuli in cat. *Brain Res.* **196**: 387–403.
92. Slawinska, U., H. Majczynski, and R. Djavadian. 2000. Recovery of hindlimb motor functions after spinal cord transection is enhanced by grafts of the embryonic raphe nuclei. *Exp. Brain Res.* **132**: 27–38.
93. Smith, M. V., A. V. Apkarian, and C. J. Hodge. 1991. Somatosensory response properties of contralaterally projecting spinothalamic and nonspinothalamic neurons in the second cervical segment of the cat. *J. Neurophysiol.* **66**: 83–102.
94. Sweet, W. H. 1991. Deafferentation syndromes in humans: A general discussion. Pages 259–290 In B. S. Nashold, Ed., *Deafferentation Pain Syndromes: Pathophysiology and Treatment*. Raven Press, New York.
95. Thompson, S. W., D. L. Bennett, B. J. Kerr, E. J. Bradbury, and S. B. McMahon. 1999. Brain-derived neurotrophic factor is an endogenous modulator of nociceptive responses in the spinal cord. *Proc. Natl. Acad. Sci. USA* **96**: 7714–7718.
96. Traub, R. J., B. J. Allen, E. Humphrey, and M. A. Ruda. 1990. Analysis of calcitonin gene-related peptide-like immunoreactivity in the cat dorsal spinal cord and dorsal root ganglia provide evidence for a multisegmental projection of nociceptive c-fiber primary afferents. *J. Comp. Neurol.* **302**: 562–574.
97. Vierck, C. J., and A. R. Light. 1999. Effects of combined hemotoxic and anterolateral spinal lesions on nociceptive sensitivity. *Pain* **83**: 447–457.
98. White, S. R., and R. S. Neuman. 1980. Facilitation of spinal motoneurone excitability by 5-hydroxytryptamine and nor-adrenaline. *Brain Res.* **188**: 119–127.
99. Whittemore, S. R., and L. A. White. 1993. Target regulation of neuronal differentiation in a temperature sensitive cell line derived from medullary raphe. *Brain Res.* **615**: 27–40.
100. Willcockson, W. S., J. M. Chung, Y. Hori, K. H. Lee, and W. D. Willis. 1984. Effects of iontophoretically released amino acids and amines on primate spinothalamic tract cells. *J. Neurosci.* **4**: 732–740.
101. Willis, W. D. 1981. Effects of peripherally and centrally administered serotonin on primate spinothalamic neurons. Pages 125–148 in B. Haber, S. Gabay, M. R. Issidorides, and S. G. A. Alivisatos, Eds., *Serotonin: Current Aspects of Neurochemistry and Function*. Plenum, New York.
102. Willis, W. D. 1982. *Progress in Sensory Physiology 3*. Springer-Verlag, New York.
103. Xu, X. M., V. Guenard, N. Kleitman, P. Aebischer, and M. B. Bunge. 1995. A combination of BDNF and NT-3 promotes supraspinal axonal regeneration into Schwann cell grafts in adult rat thoracic spinal cord. *Exp. Neurol.* **134**: 261–272 (doi: 10.1006/exnr.1995.1056).
104. Yaksh, T. L., and P. R. Wilson. 1979. Spinal serotonin terminal system mediates antinociception. *J. Pharmacol. Exp. Ther.* **208**: 446–453.
105. Ye, J. H., and J. D. Houle. 1997. Treatment of the chronically injured spinal cord with neurotrophic factors can promote axonal regeneration from supraspinal neurons. *Exp. Neurol.* **143**: 70–81 (doi:10.1006/exnr.1996.6353).
106. Yomono, H. S., H. Suzuki, and K. Yoshioka. 1992. Serotonergic fibers induce a long-lasting inhibition of monosynaptic reflex in the neonatal rat spinal cord. *Neuroscience* **47**: 521–531.
107. Young, W. 1996. Spinal cord regeneration. *Science* **273**: 451.
108. Zhou, X. F., and R. A. Rush. 1996. Endogenous brain-derived neurotrophic factor is anterogradely transported in primary sensory neurons. *Neuroscience* **74**: 945–951.