

# Transplants of Adrenal Medullary Chromaffin Cells Reduce Forelimb and Hindlimb Allodynia in a Rodent Model of Chronic Central Pain after Spinal Cord Hemisection Injury

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**In the majority of patients, spinal cord injury (SCI) results in abnormal pain syndromes in which nonnoxious stimuli become noxious (allodynia). To reduce allodynia, it would be desirable to implant a permanent biological pump such as adrenal medullary chromaffin cells (AM), which secrete catecholamines and opioid peptides, both antinociceptive substances, near the spinal cord. We tested this approach using a recently developed a mammalian SCI model of chronic central pain, which results in development of mechanical and thermal allodynia. Thirty day-old male Sprague-Dawley rats were spinally hemisected at T13 and allowed 4 weeks for recovery of locomotor function and development of allodynia. Nonimmunosuppressed injured animals received either control-striated muscle ( $n = 7$ ) or AM ( $n = 10$ ) transplants. Nociceptive behavior was tested for 4 weeks posttransplant as measured by paw withdrawals to von Frey filaments, radiant heat, and pin prick stimuli. Hemisected animals receiving AM demonstrated statistically significant reductions in both fore- and hindlimb mechanical and thermal allodynia, but not analgesia, when compared to hemisected animals receiving striated muscle transplants ( $P < 0.05$ ). Tyrosine hydroxylase immunoreactivity indicated prolonged transplant survival and production of catecholamines. HPLC analysis of cerebrospinal fluid samples from animals receiving AM transplants demonstrated statistically significant increases in levels of dopamine (sevenfold), norepinephrine (twofold), and epinephrine (threefold), compared to control values several weeks following transplant ( $P < 0.05$ ). By 28 days posttransplant, however, antinociceptive effects were diminished. These results support the therapeutic potential of transplanted AM in reducing chronic central pain following spinal cord injury.** © 2000 Academic Press

**Key Words:** hemisection; spinal cord injury; transplantation; catecholamines; adrenal medullary chromaffin cells; allodynia; chronic central pain.

## INTRODUCTION

A summary of epidemiological studies incorporating a total of 3100 spinally injured patients reveals that more than 64% suffer from chronic central pain syndromes (102, 22), so severely compromising the quality of life that suicide frequently ensues (16). In complete and partial hemisection SCI, known as Brown-Sequard syndrome in humans, reflexes return with variable motor recovery, and chronic central pain syndromes (11, 61, 7, 29), including severe persistent dysesthesias (52, 89), with concomitant changes in peripheral somatosensory responses (88) due to alterations in normal spinal circuitry. For example, abnormal pain and temperature dysesthesias develop that are evoked by somatosensory stimuli such as joint movement and pressure (89).

These syndromes remain refractory to conventional pain treatments and require novel therapeutic approaches. Narcotic analgesics are sedative and cause serious side-effects, and epidural and subarachnoid delivery systems introduce a variety of additional health problems such as mechanical malfunction of pumping devices and infection of tunneled catheters and injection ports (57). In addition, periodic surgical procedures are required for maintenance and pump replacements. Thus, one-time transplantation of nonproliferative cells that secrete antinociceptive substances are an attractive means of eliminating these problems (10, 27, 98, 99, 34, 35). Also, secreted compounds acting in a site-specific manner allow precise targeting and selective exposure. Adrenal medullary chromaffin cells (AM) secrete a "trophic cocktail" of therapeutic agents (86, 49), including catecholamines and opioid peptides (54, 96, 40). When injected into the spinal intrathecal space in mammalian models of acute and chronic pain they significantly reduce pain without tolerance (74, 12, 104, 105). Successes with AM transplants have

been realized in cancer patients with chronic pain (97, 73, 15, 64, 9).

Our group has developed a model of chronic central pain equivalent to the Brown–Sequard syndrome (22), where somatosensory changes occur such that normally nonnoxious stimuli elicit behavioral changes consistent with receipt of noxious stimuli and persist for the life of the animal bilaterally, rostral and caudal to the lesion. Following SCI, spinal pain fibers sprout or reorganize (55, 56), creating an aberrant circuit leading to abnormal pain sensations (23) and hyperexcitability of dorsal horn neurons (22). Behaviorally, the presence of ongoing, spontaneous pain can be measured indirectly by changes in response thresholds to formerly nonnoxious peripheral stimuli (88), providing objective measures of chronic pain. In addition, threshold changes are accompanied by changes in whole body posturing, vocalizations, writhing, and other behaviors consistent with experience of nociceptive stimuli.

Due to their ability to attenuate persistent pain in peripheral neuropathic models, we wanted to test the hypothesis that intrathecal transplantation of AM would reduce mechanical and thermal allodynia that develops following spinal cord hemisection injury in rodents. A preliminary report of these data has been reported elsewhere in abstract form (43).

## MATERIALS AND METHODS

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.

### *Surgical Procedures*

**Hemisection injury.** Male Sprague–Dawley rats, 100–125 g, were deeply anesthetized by intraperitoneal injection of sodium pentobarbitol (40 mg/kg), and the left side of the spinal cord was hemisectioned 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 located by finding the last rib, which attaches to the rostral end of the T13 vertebrae. The surgical field was shaved and prepared with Betadine, and a longitudinal incision was made exposing several segments. A laminectomy was performed at T11, the lumbar spinal cord was identified with the accompanying dorsal vessel, and the spinal cord hemisectioned at T13, with a no. 11 scalpel blade without damage to the dorsal spinal artery or branches. Iridectomy scissors were used to ensure the completeness of the hemisection. Muscle and fascia were sutured closed 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 s.c.) for rehydration and penicillin-G (0.35 ml/kg i.m.) (Wyeth Laboratories, Philadelphia, PA) as a prophylactic antibiotic. Following surgery, animals were maintained under the same preoperative conditions, fed *ad libitum*, and were eating and drinking within 3 h after surgery. Weight loss was minimal, occurred acutely over the first two postoperative days, and was not greater than 5% of the total body weight.

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. Locomotor function was observed and recorded using the BBB Locomotor Rating Scale (5) to ensure that a motor recovery of the ipsilateral limb occurred and did not impair the somatosensory behavioral tests. Animals that demonstrated loss of locomotion in both hindlimbs, indicating bilateral corticospinal tract transection, were excluded from the study.

Five groups of rats were compared: (1) normal ( $n = 10$ ), (2) hemisectioned alone ( $n = 15$ ), (3) sham hemisectioned in which the surgical procedure included cutting the dura but no spinal lesion was made ( $n = 7$ ), (4) hemisectioned with adrenal medullary transplants (HS + AM) ( $n = 10$ ), and (5) hemisectioned with striated muscle transplants (HS + SM) ( $n = 7$ ). No behavioral difference was seen in normal and sham hemisectioned groups in presurgical compared to postsurgical behavioral tests: locomotor scores remained at 21 for these two groups and there was no change in somatosensory responses. These groups will not be discussed further. The hemisectioned-alone group was not statistically different compared to the results of the hemisectioned group with striated muscle transplants. We chose to present data only from the HS + AM group for comparison to the HS + SM group.

**Adrenal medullary chromaffin cell and striated muscle transplantation.** On postsurgical day 30, graft recipients and donors were deeply anesthetized as described previously. Heterologous allograft donors were adult males from the same strain, and were age, sex, and weight matched to the recipients. Bilateral abdominal incisions were made and both adrenal glands from donor animals were removed. Donor adrenal medullae tissue was rapidly dissected free of cortical tissue from two adrenal glands and minced into pieces less than 0.5 cm<sup>3</sup> in ice-cold sterile Hanks Balanced Salt Solution (Gibco, Grand Island, NY) under a dissecting microscope. Control animals received equal volumes of heterologous striated rectus abdominis skeletal muscle allografts from donor animals.

The spinal cord of recipient animals was exposed by a laminectomy at the lumbar enlargement and the dura incised 2–3 mm for intrathecal graft placement of AM or muscle tissue. Minced tissue was drawn into

polyethylene PE50 tubing that was attached to a 50- $\mu$ l Hamilton syringe. The PE50 assembly was inserted through a dural incision, advanced 5 mm, and the tissue was injected into the intrathecal space at L1. A laminectomy-fitted piece of Parafilm M paraffin wax was placed over the injection site. Muscle and fascia were sutured closed and the animals were treated postoperatively as described previously.

### *Behavioral Assays*

Rats were housed with a reversed light/dark cycle of 16 h/8 h, where the dark cycle began at 7:00 a.m. Since the rats are nocturnal animals, the behavioral tests occurred during the dark cycle or their "awake" period in the circadian rhythm.

*BBB locomotor scoring.* To ensure that motor recovery occurred and did not impair somatosensory behavioral tests, locomotor function was observed for 28 days following hemisection injury and recorded using the modified open field test first developed by Tarlov and Klinger (84) and recently modified into the Basso, Beattie, and Bresnahan (BBB) Locomotor Rating Scale (5). The score is based on locomotor ability following experimental SCI in rodent models. Briefly, the BBB is a 21-point ordinal scale ranging from 0, which is no discernable hindlimb movement, to 21, which reflects 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 the return of isolated joint movements of the three joints (hip, knee, ankle), scores from 8 to 13 describe the intermediate recovery phase with the return of paw placement, stepping, and forelimb-hindlimb coordination, and scores from 14 to 21 rank the late phase of recovery with the return of toe clearance during the step phase, predominant paw position, trunk stability, and tail position. The score was tabulated and considered to be an indicator of motor recovery after spinal cord hemisection. Animals with observable contralateral hindlimb impairment were excluded from the study.

*Mechanical allodynia—von Frey and pin.* A blinded observer performed behavioral tests examining mechanical and thermal allodynia, preoperatively and postoperatively, for both forelimbs and hindlimbs. Prior to testing, all animals were environmentally acclimated to the clear Plexiglass cubicle testing apparatus (8  $\times$  8  $\times$  18 cm) for 4 h daily for 3 days. Preoperative testing began 3 days prior to surgery to establish both individual and group baseline behaviors. The tests were performed postoperatively every 7 days 4 weeks prior to transplantation, after which animals were tested one or two times per week. Since no significant side-to-side differences were found, data from each limb were collapsed into a combined fore- and hindlimb score for ease of comparison. Mechanical allodynia of the glabrous skin of the paw was quantified

by measuring the number of brisk paw withdrawals in response to normally innocuous or subthreshold mechanical stimuli (20). The subthreshold mechanical stimuli were von Frey filaments (Stoelting, Wood Dale, IL) with bending forces of 4.78 and 9.96 mN. In addition, suprathreshold mechanical stimuli, a von Frey filament with a bending force of 204.1 mN, and a mechanical noxious stimulus, a pin, were used. In human perceptual terms, these mechanical forces are perceived as a subthreshold stimulus, light touch, poke, and noxious prick, respectively. The pin did not cause overt tissue damage and was selected to control for sedation of responses to normally noxious stimuli (analgesia).

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 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 and this value was normalized to the prehemisection baseline at the level of the individual animal. Data were analyzed and graphed as group data, where prehemisection baseline is displayed as zero, and were compared as change relative to the baseline pre- and posthemisection and transplant responses for between group comparisons.

*Thermal allodynia.* Thermal allodynia was measured by the latency of paw withdrawal to noxious stimuli as previously described by Bennett and Xie (6) and Hargreaves *et al.* (45). Animals were placed in Plexiglas boxes on an elevated glass plate under which a light box was applied. A radiant heat stimulus was applied by concentrating 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 light and paw withdrawal. Time was defined as the paw withdrawal latency. Five minutes were allowed between each trial and three trials were averaged for each limb, which were then combined. The results for thermal tests were then normalized to the prehemisection baseline at the level of each individual animal. These data were analyzed and graphed as group data, where prehemisection baseline is displayed as zero, and the data were compared as a change relative to the baseline pre- and posthemisection and transplant responses for between group comparisons.

### *High Pressure Liquid Chromatography*

To directly measure the concentrations of dopamine (DA), norepinephrine (NE), and epinephrine (E), high

pressure liquid chromatography with electrochemical detection (HPLC) was performed on cerebrospinal fluid (CSF) obtained from rats undergoing behavioral testing that received either AM ( $n = 7$ ) or control striated muscle cell transplants ( $n = 7$ ) 30 days posttransplant. Under deep anesthesia with sodium pentobarbital (40 mg/kg i.p.) dual laminectomies and dural incisions were made, and polyethylene PE10 tubing was attached to dual 250- $\mu$ l Hamilton syringes threaded approximately 1 cm into the subdural spaces both rostral and caudal to the transplant site. The inflow catheter was perfused with 75  $\mu$ l artificial CSF (in mM): NaCl, 126.5; NaHCO<sub>3</sub>, 27.5; KCl, 2.4; KH<sub>2</sub>PO<sub>4</sub>, 0.5; CaCl<sub>2</sub>, 1.1; MgCl<sub>2</sub>, 0.85; Na<sub>2</sub>SO<sub>4</sub>, 0.5; glucose, 5.9, adjusted to pH 7.3 with an O<sub>2</sub>/CO<sub>2</sub> mixture, 95% v/v. After 30 s, CSF perfusate was collected through the outflow catheter assembly and 1N perchloric acid was added (10% v/v) to prevent DA degradation and induce protein precipitation. Samples were then stored frozen at -70°C for later analysis. Catecholamine production was assayed via techniques modified from Yaksh and Tyce (101). Samples 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 1.5 mM sodium octane sulfonic acid, 75 mM NaH<sub>2</sub>PO<sub>4</sub>, triethylamine, and 10% acetonitrile dissolved in water at pH 3.0. Concentrations of DA, NE, and E in animals receiving either chromaffin or striated muscle cell transplants were calculated from relative peak heights to standard sample concentrations. CSF catecholamine levels were determined for each animal and means were calculated based on all animals within a group.

### *Immunohistochemistry*

At 30 days posttransplant, animals receiving chromaffin cells were deeply anesthetized with sodium pentobarbital and perfused intracardially with heparinized 0.9% saline followed by 4% cold buffered paraformaldehyde. After perfusion, spinal cords were fixed and stained for tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine biosynthesis, to assess transplant integrity and location (78), with positive TH immunoreactivity, indicating transplant survival and catecholamine synthesis. After removal from the vertebral column, spinal cords were fixed for 24 h, at 4°C before the solution was replaced with serial increments leading to 30% sucrose. Sections were cryosectioned at 20  $\mu$ m at -20°C and, following preincubation in normal horse serum, reacted for 12 h at room temperature with polyclonal mouse TH antibody (Instar 1:500, Stillwater, MN). Secondary antibody, biotinylated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA), was applied for 90 min at room tem-

perature followed by ABC-HRP Vector kit reagents. The reaction was completed using diaminobenzidine with 0.01% H<sub>2</sub>O<sub>2</sub>. Tissue was then cleared and mounted in Permount (Fisher, Fair Lawn, NJ), after antibody staining for visualization and photomicroscopy attached to an Olympus BH-2 microscope with a PM-10ADS camera mount and C-35AD-2 camera.

### *Statistical Analysis*

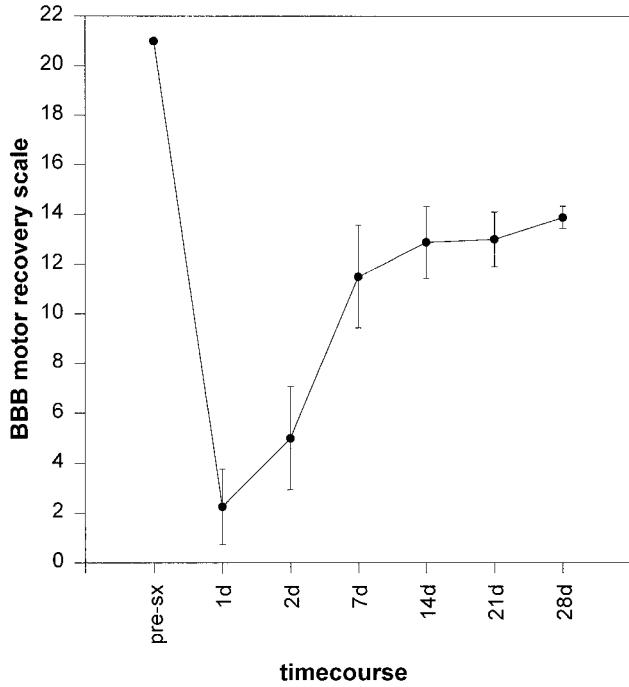
All statistical tests were evaluated at the alpha level of significance of 0.05 by two-tailed analyses. The data from these procedures were tested for statistical significance using ANOVA and Friedman's rank test using one-way repeated measures. This was followed by tests of factors, including pair-wise comparisons where appropriate with either the paired Student's *t* test (comparisons of behavioral test results before and after hemisection surgery or comparison before and after cellular transplantation within each animal) or the two sample Student's *t* test (for between group comparisons). All data management and statistical analyses were performed using Jandel SigmaStat (v1.0). All values are graphed using Jandel SigmaPlot (v5.0) as means ( $\bar{X}$ )  $\pm$  standard error (SEM).

## RESULTS

### *Behavioral Measures*

*Recovery of locomotor function pursuant to behavioral analysis.* All animals started with baseline hindlimb BBB scores of 21 (Fig. 1). T13 spinal hemisection resulted in ipsilateral hindlimb paralysis in all animals. One day after surgery, the animals scored  $2.25 \pm 0.52$  for the ipsilateral hindlimb which translates as movement possible in only one joint and no locomotor function. No BBB changes were observed in the contralateral hindlimb. By postsurgical day 7, animals recovered considerable ipsilateral motor function ( $11.5 \pm 2.06$ ), allowing for frequent to consistent weight supported steps and frequent forelimb-hindlimb coordination. Ipsilateral BBB scores increased slightly at postsurgical days 14 through 28 from  $12.88 \pm 1.43$  to  $13.88 \pm 0.44$ , and remained at similar values for the remainder of the study. With a score of 12-13, the rat is able to produce consistent weight supported steps and gait and consistent forelimb-hindlimb coordination, with frequent toe drags and rotated paw position at initial contact and lift-off. This plateau of recovery allows for full and complete behavioral testing of somatosensory-induced paw withdrawal.

*Mechanical allodynia and pin.* At 28 days, spinal hemisection produced a statistically significant ( $P < 0.05$ ) increase in paw withdrawals of  $2.40 \pm 0.17$  and  $1.33 \pm 0.19$  (forelimbs), and  $3.57 \pm 0.23$  and  $1.67 \pm 0.17$  (hindlimbs), respectively, to low and medium

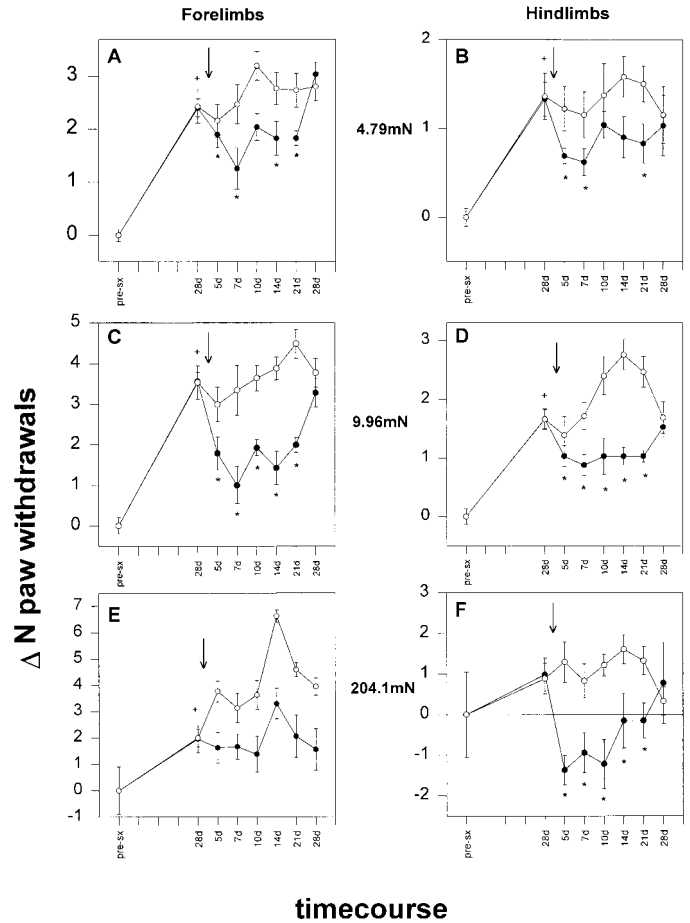


**FIG. 1.** BBB motor scores for ipsilateral hindlimbs of hemisected animals plotted in days (d) after hemisection surgery displayed as means  $\pm$  standard errors. Twenty-one represents normal locomotion, zero represents no observable movement. On postsurgical day 1, the animals scored a  $2.25 \pm 1.52$ , by day 7 motor scores had increased significantly with plateau of  $12.88 \pm 1.43$  at day 14 with little variation for the remainder of the study (pre-sx, presurgical value).

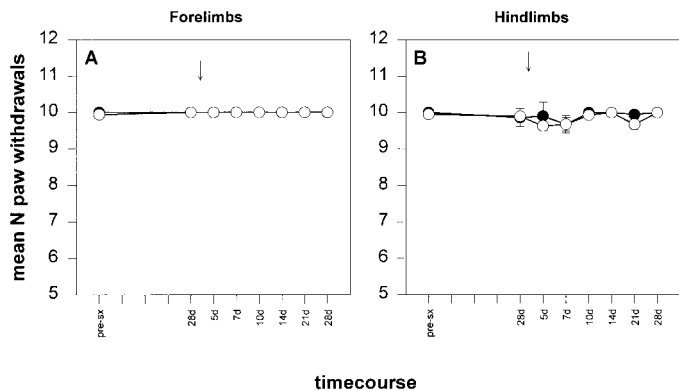
threshold von Frey filaments, 4.79 and 9.96 mN, compared to a baseline of 0 (Figs. 2A–2D) in pretransplant HS + AM animals. Pretransplant HS + SM control animals also developed a significant ( $P < 0.05$ ) allodynia with 4.78 and 9.96 mN filaments:  $2.43 \pm 0.32$  and  $3.54 \pm 0.41$  (forelimbs), and  $1.36 \pm 0.26$  and  $1.66 \pm 0.17$  (hindlimbs). Transplantation of AM created statistically significant decreases in the paw withdrawals when compared to pretransplant values ( $P < 0.05$ ). HS-AM paw withdrawals often did not vary significantly when compared to presurgical baseline values. The reduction in paw withdrawal was significantly different between HS + SM and HS + AM groups ( $P < 0.05$ ). Posttransplant time points demonstrate paw withdrawal decreases of up to 72% in chromaffin cell recipients when compared to animals in chronic pain states. In contrast, responses for control striated muscle transplants demonstrated no statistically significant changes from postinjury values. In addition, HS + SM animals often developed further mechanical allodynia over time, up to  $3.20 \pm 0.27$  from  $2.43 \pm 0.32$  (forelimbs) and  $1.58 \pm 0.23$  from  $1.36 \pm 0.26$  (hindlimbs) for the 4.79 mN stimulus, and  $4.49 \pm 0.35$  from  $3.54 \pm 0.41$  (forelimbs) and  $2.76 \pm 0.26$  from  $1.66 \pm 0.17$  (hindlimbs) for the 9.96 mN stimulus, respectively. In the 4.78 and 9.96 mN groups antinociceptive

effects of chromaffin cell transplants were strongest at 7 days posttransplant, with a mean reduction of  $5.50 \pm 0.62$ . In almost all groups receiving AM transplants, antinociceptive effects diminished over time.

The pattern of antinociception for the high threshold, 204.1 mN, von Frey filament was very similar to that seen for the low and medium intensity filaments (Figs. 2E and 2F). The development of mechanical allodynia was not as profound due to a higher number of baseline paw withdrawals, indicating that the stimulus was more detectable before hemisection. At this



**FIG. 2.** Departure from baseline of mean number of paw withdrawals of fore- and hindlimbs plotted in days (d) after hemisection surgery to various strength von Frey mechanical stimuli for the hemisected animals receiving AM transplants (filled circles) or control transplants of striated muscle tissue (open circles) displayed as means  $\pm$  standard errors. Von Frey stimulus strengths are displayed by the milliNewtons (mN) bending force generated. Transplantation (arrow) of AM led to significant reduction ( $*P < 0.05$  posttransplant) in paw withdrawals when compared to controls after hemisection injury ( $+P < 0.05$ ). These values were statistically different from pretransplant and controls in both fore- and hindlimb values for both the 4.79 mN (A, B) and 9.96 mN (C, D) stimuli, respectively. The 204.1 mN strength von Frey responses also demonstrated increased responsiveness to hemisection and AM, but not control transplants, reduced both fore- and hindlimb paw withdrawals to nocigenic stimulation (E, F).



**FIG. 3.** Mean number of paw withdrawals of fore- and hindlimbs of animals receiving either AM (filled circles) or control striated muscle (open circles) transplants to pinprick stimulus displayed as means  $\pm$  standard errors. Nocigenic stimuli was equally aversive to all animals and was not affected by either transplantation (arrow) of AM or control striated muscle tissue after hemisection injury indicating preservation of normally noxious responses.

stimulus strength, the development of chronic central pain is not statistically significant, however, fore- and hindlimb withdrawals increased to  $1.95 \pm 0.52$  and  $0.99 \pm 0.41$  in HS + AM animals, and up to  $1.99 \pm 0.33$  and  $0.89 \pm 0.38$  in HS + SM animals, respectively. AM, unlike control muscle transplants, did reduce pain-related behaviors following transplantation, from  $1.37 \pm 0.68$  and  $-1.37 \pm 0.36$  (a decrease of 23.6%) in fore- and hindlimbs, respectively. As with other strength von Frey filaments, this effect was observed first at 7 days following transplantation as mean number of paw withdrawals approached or surpassed control levels.

For the pinprick test, responses were maximal at baseline (10 out of 10), and hemisection did not alter the response to this stimulus (Fig. 3). AM transplants did not result in any statistically significant differences in pinprick response incidence at any time point and between the HS + AM or HS + SM groups.

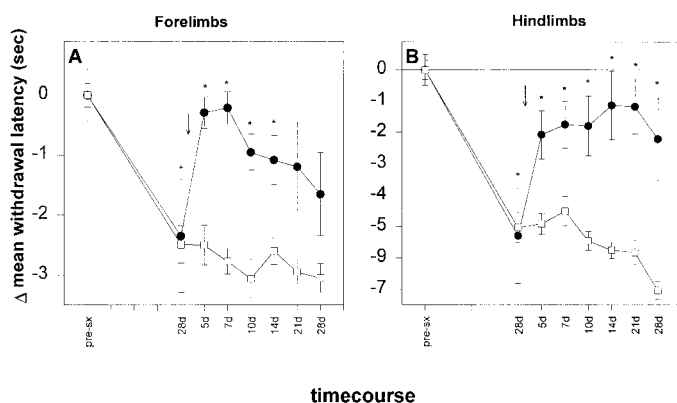
**Thermal allodynia.** Spinal hemisection resulted in paw withdrawal latencies that were significantly ( $P < 0.05$ ) decreased when compared to presurgical values for both fore- and hindlimbs (Figs. 4A and 4B). Pre-transplant fore- and hindlimb latencies in hemisections, in animals to receive chromaffin or muscle transplants, were  $-2.35 \pm 0.95$  and  $-5.29 \pm 1.52$ , and  $-2.49 \pm 0.31$  and  $-5.03 \pm 0.48$  s, respectively. Thus, limb withdrawals occurred at a shorter latency and consequently at a lower temperature.

AM transplants changed both fore- and hindlimb responses such that paw withdrawal latencies for the HS + AM group were significantly decreased (to  $-0.29 \pm 0.26$  and  $-2.07 \pm 0.77$  s) compared to the HS + SM group which had no significant increase. The HS + AM forelimb withdrawal latency was not significantly different compared to baseline, which reflected

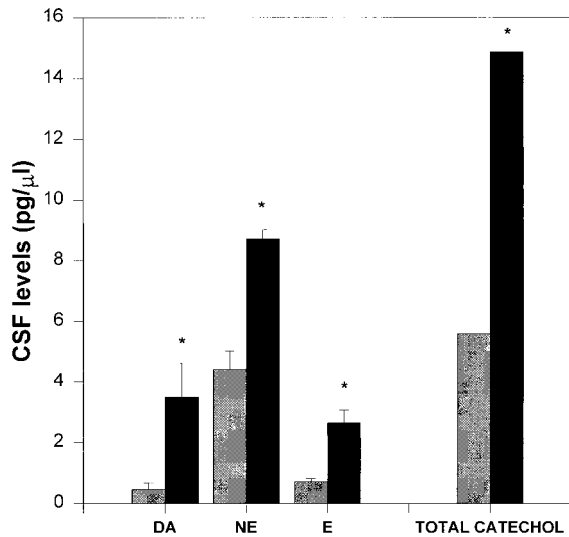
as much as a 91.0% decrease compared to pretransplant values. For the hindlimbs, chromaffin cell transplants resulted in responses that were also antinociceptive, with posttransplant values reaching a 91.4% decrease when compared to animals in chronic pain states, also not significantly different from preinjury values. As with the von Frey mechanical data, HS + AM values trended toward pretransplant values and toward values of the control group (HS + SM) in both fore- and hindlimb withdrawal latencies at 28 days posttransplant. These values approximated baseline more closely than with mechanical stimuli, indicating a stronger antinociceptive effect in this behavioral paradigm.

### High Pressure Liquid Chromatography

CSF concentrations of catecholamines (DA, NE, and E) in samples taken from animals transplanted with either AM or control striated muscle transplants are shown in Fig. 5. The mean DA concentrations released into the CSF of HS + SM animals was  $0.46 \pm 0.22$  pg/ $\mu$ l, while NE and E were  $4.42 \pm 0.61$  and  $0.72 \pm 0.1$  pg/ $\mu$ l, respectively. CSF concentrations of all three catecholamines were significantly increased in HS + AM animals when compared to HS + SM animals ( $P < 0.05$ ). DA, with a level of  $3.5 \pm 1.13$  pg/ $\mu$ l, was increased 760.8% above normal; NE, with a level of  $8.72 \pm 0.31$  pg/ $\mu$ l, was increased 197.2% above normal; and E, with a level of  $2.66 \pm 0.41$  pg/ $\mu$ l, was increased 369.4% above normal. The increase in total CSF catecholamines by transplantation of AM was calculated to be approximately 265.7%. Thus, chromaffin cells secrete catecholaminergic substances into the CSF as



**FIG. 4.** Mean change from baseline of paw withdrawal latency to noxious heat stimulus for fore- and hindlimbs of animals receiving (arrow) either AM (filled circles) or control striated muscle transplants (open circles) plotted in seconds. Forelimb (A) and hindlimb (B) responses to radiant heat stimuli displayed as means  $\pm$  standard errors (\* $P < 0.05$  postsurgical; \* $P < 0.05$  posttransplant). Transplants of AM but not striated muscle tissue increased the withdrawal latency to near-presurgical levels in the fore- and hindlimbs indicating reduction of thermal allodynia.



**FIG. 5.** HPLC analysis of CSF 30-days posttransplant shows that AM (dark bars) but not control striated muscle (light bars) transplants secrete catecholaminergic substances intrathecally as indicated by elevated levels of dopamine (DA), norepinephrine (NE), epinephrine (E), and total catecholamines (\* $P < 0.05$  significant difference between groups).

indicated by elevated levels of DA, NE, and E 30 days following transplantation.

#### Immunohistochemistry

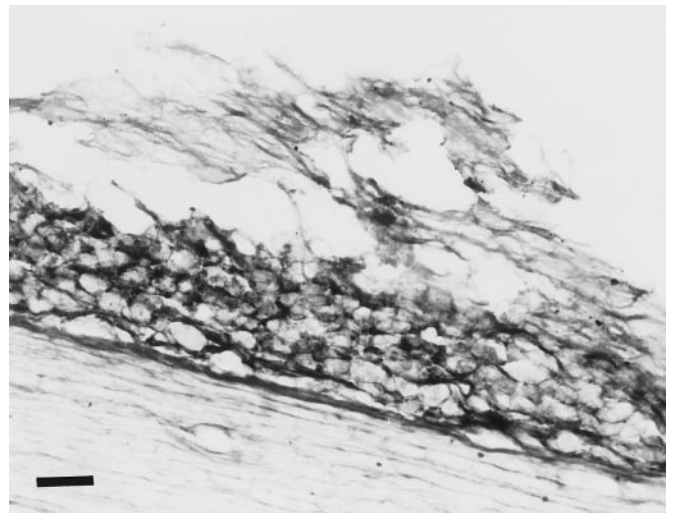
At 30 days posttransplantation, subdural grafts of adrenal medullary tissue are easily visualized dorsal to the spinal cord in the intrathecal space below the dura mater at the original site of transplantation indicating no migration. Additionally, we observed no gross infiltration by the transplant into the host spinal cord parenchyma. Chromaffin cells in the transplant remained in tight clusters and generally retained the polyhedral shape normally found in the *in situ* adrenal medulla. At 30 days posttransplantation numerous TH-positive cells were present in the intrathecal space (Fig. 6). No adverse anatomical effects, such as cord compression or cellular distortion of the spinal cord proper, and no indication of tumorigenesis by the chromaffin cell transplant mass were observed.

#### DISCUSSION

In this study, we confirm that animals receiving thoracic spinal hemisection recovered ipsilateral locomotor function and developed bilateral rostral and caudal mechanical and thermal allodynia, consistent with chronic central pain syndromes (21, 22). Tests of allodynia evoked nocifensive behaviors, in agreement with altered thresholds to non-noxious stimulation, which were well established by 28 days. Transplantation of AM resulted in behavior that was not statistically different when compared to presurgical behavioral val-

ues, while groups that received muscle transplants demonstrated no reduction in allodynia. Additionally, there was no undesirable analgesia as animals still responded to normally noxious stimuli. As determined by HPLC and immunocytochemical measurements, chromaffin cells survive and secrete catecholamines *in vivo* for at least 28 days; however, the behavioral effects of chromaffin cell transplants were diminished by this timepoint. Thus, the data presented in this study support the hypothesis that AM transplants attenuate mechanical and thermal allodynia that develop after SCI in the hemisection model of chronic central pain.

Few mammalian models exist to explore SCI pain, and in most, variable lesion characteristics lead to differences in recovery and development of allodynia (103). Spinal contusion lesions may better parallel human SCI (14); however, the hemisection model has the advantages of consistency, surgical selectivity, avoiding variability of different lesions, ease of reproducibility, and avoidance of profound hindlimb locomotor deficits that occur after contusion. Our behavioral data confirm and extend results of AM transplants in other models of neuropathic pain (74, 12, 104, 105); however, only two other models of central neuropathic pain have examined antinociceptive efficacy of AM transplantation. With the quisqualate model (12), the authors report reduction of mechanical and cold allodynia; however, significance was only demonstrated when data were presented as cumulative means. Yu *et al.* (105), using a photochemical injury paradigm, report significant reductions in mechanical and cold allodynia 2



**FIG. 6.** Longitudinal section showing spinal white matter with TH-positive chromaffin cell transplant situated under dura mater. At 30 days posttransplant, AM chromaffin cell tissue is easily visualized dorsal to the spinal cord in a discrete mass at the original transplant site. Tyrosine hydroxylase (TH) immunoreactivity indicates survival and biosynthetic activity of intrathecally transplanted chromaffin cells as TH is the rate-limiting enzyme in catecholamine biosynthesis. Scale bar, 50  $\mu$ m.

weeks following bovine AM transplantation, but with reduced effectiveness by 8 weeks (105). Our findings confirm the antinociceptive effects of AM with a model that demonstrates robust bilateral fore- and hindlimb allodynic responsiveness after SCI and a statistically significant antinociception due to transplant-induced changes occurring locally in the region of injury/transplant that produce distant beneficial alterations in spinal nociceptive circuitry.

Since our model of chronic pain demonstrates unexpected changes bilaterally and at a distance above and below the lesion, a careful consideration of spinal circuitry is needed to clarify these findings. Brown-Sequard syndrome is classically characterized by ipsilateral hemiparalysis and contralateral hypalgesia (13), but hemisection also results in severe chronic pain bilaterally below the lesion (42, 69) due to changes in contralaterally projecting primary dorsal horn nociceptive afferent fibers (53, 24). Additionally, our laboratories and others have shown bilateral sprouting of C-fiber primary afferents at levels rostral and caudal to spinal lesions (55, 56, 21). The clinical corollary is bilateral alteration of sensory processing as occurs in spinally injured patients—not necessarily dependent upon the sidedness of SCI but on the tracts and cellular processes altered.

After hemisection anatomical substrates remain in place for nociceptive transmission within remaining and/or modified spinal cord circuitry bilaterally, rostrally, caudally, and through the intact cord adjacent to the lesion site (94, 85). This circuitry supports the development of allodynia outside of the conventionally accepted (hemisected) pathways. Ascending pathways include the spinothalamic tract, which mediates both intact ipsilateral and contralateral nociception caudal to the lesioned segment (95), the uninterrupted spinocervicothalamic tract (33, 82), and the spinoreticular tract (75), which mediate somatosensation bilaterally. Pain and temperature information are also transmitted rostrally by the ipsilateral dorsal column as primary afferent C-fibers are found within the dorsal funiculus (65).

Current observations of fore- and hindlimb responses to local circuit stimuli such as crossed flexor-reflex responses, as recorded behaviorally and electrophysiologically by Sherrington (77) and Grillner (41), are not novel concepts. Noxious stimulation is able to produce a heterosegmental response mediated by local spinal (66) and supraspinal mechanisms (70). The spinal cord contains an extensive network of propriospinal connections characterized by redundantly organized reciprocal side-to-side projections (26) via heterosegmental, intersegmental, and supraspinal pathways (59, 1, 30). Long cervical propriospinal neurons project to far caudal lumbar segments (81), and complimentary bi- and unilateral lumbosacro-cervical connections (60) exist. These neurons originating in the fore-

or hindlimb segments directly synapse onto interneurons involved in nociceptive and cutaneous reflexive pathways of hind- and forelimbs (4, 81, 48).

With this circuitry in place, changes responsible for initiation of both fore- and hindlimb allodynia can take place at a singular location and utilize many pathways for transmittal of nociceptive information. It is thus reasonable to hypothesize that since initial alterations occurring at the site of injury confer changes at multiple loci, transplantation can and do lead to widespread changes in spinal circuitry (46) as well.

The rationale to pursue catecholamines as antinociceptive in chronic central pain syndromes is that spinal catecholaminergic terminal systems have been shown to mediate antinociception behaviorally and anatomically (68, 92). Specifically, the superficial dorsal horn of the spinal cord contains high DA receptor densities (31),  $\alpha$ -adrenergic receptors (83), and E-containing axonal profiles from neurons that descend from the caudal brainstem (17), a known inhibitory pathway (95). Axonal boutons of catecholaminergic neurons directly contact somata and/or dendrites of lamina I, IV, and V spinothalamic tract (STT) wide dynamic range and high-threshold projection neurons (91, 92). Furthermore, iontophoretic application (93), superfusion (62), and intrathecal injection (100) of catecholamines onto STT cells results in hyperpolarization of substantia gelatinosa neurons, an antinociceptive effect reversed by intrathecal adrenergic antagonists (100, 36).

Chromaffin cells survive for long terms and are rich sources of catecholamines (27)—making them excellent candidates for transplant delivery systems. They secrete compounds that are thought to act individually in a subthreshold fashion, producing antinociception via synergistic action (27), and also secrete the opioid peptides, met- and leu-enkephalin (18, 71), which may further contribute to the reduction of pain-related behaviors. Studies using the  $\alpha$ -adrenergic antagonist phentolamine and the opioid antagonist naloxone reduced the antinociceptive properties of AM transplants (90, 79). Mechanisms of antinociception include the attenuation of NMDA-mediated hypersensitivity (44, 78) and/or neurotrophic promotion of survival of inhibitory dorsal horn GABAergic neurons (47).

A remarkable finding in this study is that lumbar transplants mediate changes in forelimb behaviors. It is doubtful that lumbar transplants could affect forelimb behaviors via rostral diffusion of compounds. CSF movement within the spinal canal exerts significant effects on the drug distribution; that is, CSF produced in the brain reaches the spinal cord by rostral-caudal flow along the dorsal aspect of the cord to the sacral cul de sac and then flows rostrally along the ventral cord surface to the basal cisterns (19). This progression is faster than the subarachnoidal ascent toward the convexity of the brain. Substances secreted by transplants must upwardly diffuse against this flow to reach the

cervical enlargement, a process that is further antagonized by scar tissue formed at the injury site just rostral to the transplant. Furthermore, even though agents delivered onto the spinal surface move down a concentration gradient by simple Brownian motion and partition into microenvironments of thermodynamic stability, they must evade catabolism and uptake along the way to diffuse through transplant material, pia, white matter and gray extracellular parenchyma. These hurdles must be cleared in order for secreted substances to reach the specific neuronal or receptor population being targeted. It is also unlikely that compounds are being taken up by local vasculature and redistributed to cervical levels as epinephrine-induced vasoconstriction reduces flow through the epidural venous plexus which in turn would decrease drug uptake into systemic circulation (25, 8). Thus, due to these multiple challenges, it is most likely that antinociception in both fore- and hindlimbs is affected via modulation of local lumbar neural circuitry.

Analysis of CSF catecholamines gives insight into AM secretory activity and the DA → NE → E biosynthetic pathway. We report normal relative CSF catecholamine concentration ratios of approximately 1:10:1 for DA:NE:E, consistent with rodents and other species (50, 72), but within the adrenal gland, medullary tissue produces NE and E in a ratio of 3:7 (87, 32). The conversion of NE to E occurs *in situ* since the steroid-containing adrenal cortex influences phenylethanolamine *N*-methyltransferase activity in the medulla, resulting in elevated and reduced levels of E and NE, respectively (67, 32). Thus, in the absence of adrenal cortical influence, ratios of NE:E in the transplant are altered; thus our result of a posttransplant 3:1 NE:E CSF ratio is not unexpected. Only one other AM transplant report includes CSF analysis, which reports a 1:4 NE:E ratio (72). These discrepancies may be due to differences in dissection technique or secretory activity of the transplants since some cells preferentially produce either NE or E (32).

An additional concern is that tissue transplanted to the pial cord surface is subject to host/graft rejection, contributing to a temporal loss of antinociception. In contrast to the widespread belief that the CNS is an immunologically privileged site, transplantation of AM tissue into the brain (37–39) and spinal cord (28) has demonstrated that this privilege is not absolute. Others have demonstrated prolonged graft survival for up to 1 year with a short-course regimen of cyclosporin-A (63, 76), purportedly due to evasion of immune surveillance. While our study demonstrates continued catecholamine secretion into the CSF, this was insufficient to reduce allodynia past 28 days (see also 104). In the absence of immunosuppression, we suspect that this reduction in antinociception is due to slow host rejection of AM cells required for perceptible behavioral effects. In other studies, isolated bovine medullary

cells demonstrated no proliferative response in an *in vitro* lymphocyte proliferation assay (27, 28, 58), thus other nonchromaffin medullary passenger cells may stimulate an immune response. In lieu of immunosuppression, Aebischer *et al.* (2) have developed a technique in which xenogenic chromaffin cells are encapsulated within a semipermeable polymer shell prior to implantation. This approach overcomes potential immunological rejection by providing a barrier between transplant and host immune system. An alternate explanation of the loss of antinociceptive effect is that tolerance to the secreted substances developed; however, we do not think this is the case as AM-secreted histogranins have been shown to prevent the development of tolerance (79).

In summary, chronic central pain syndromes are characterized by the presence of persistent dysesthetic pain (52, 51) with concomitant changes in peripheral somatosensory responses (88). Our data in a spinal cord injury model of chronic central pain demonstrate behavior consistent with a significant decrease in mechanical and thermal allodynia in response to peripheral stimuli bilaterally in both forelimbs and hindlimbs after AM transplantation without sedation or loss of normal somatosensation. Using TH immunoreactivity and HPLC, the anatomical location and status of the cells was determined and survival of intrathecally transplanted chromaffin cells with active biosynthesis of catecholamines indicated. There is clinical precedence for the successful application of AM transplant therapy in treatment of cancer pain (97, 73, 15, 64). We suggest this or AM autografts which have been expanded in tissue culture (106) may be useful in treatment of chronic pain following spinal cord injury as well.

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