

Changes in Serotonin, Serotonin Transporter Expression and Serotonin Denervation Supersensitivity: Involvement in Chronic Central Pain after Spinal Hemisection in the Rat

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Spinal cord injury (SCI) results in abnormal locomotor and pain syndromes in humans. In a rodent SCI model, T13 unilateral spinal hemisection results in bilateral mechanical allodynia and thermal hyperalgesia, partly by interruption of tonic descending serotonin (5-HT) inhibition. In the current study, we examined changes in density and distribution of 5-HT and 5-HT_T in cervical (C8) and lumbar (L5) enlargements after T13 spinal hemisection and studied the effects of intrathecally delivered 5-HT (10, 21, and 63 μg), 5-HT antagonist methysergide (125 μg/kg), and 5-HT reuptake inhibitor fluvoxamine (75 μg/kg) on pain-related behaviors. Thirty-day-old male Sprague–Dawley rats were spinally hemisected and sacrificed at 3 (*n* = 20) and 28 (*n* = 20) days postsurgery for immunohistochemistry, Western blot, and ELISA analysis and compared against sham-operated animals (*n* = 10). At day 3, C8 5-HT levels were not significantly changed but at L5 there was a significant decrease in ipsilateral 5-HT in laminae I–II followed by incomplete recovery at 28 days postinjury. At both 3 and 28 days postinjury, C8 5-HT_T levels were not significantly changed, but at L5 there was significant ipsilateral up-regulation of 5-HT_T in laminae I–II. A second group of animals (*n* = 30) was hemisected and, starting at 28 days postinjury, behaviorally tested with intrathecal compounds. Increasing doses of 5-HT attenuated both fore- and hindlimb mechanical allodynia and thermal hyperalgesia, and effects of endogenous 5-HT were attenuated by methysergide and enhanced with fluvoxamine, all without locomotor alterations. Sham controls (*n* = 10) were unaffected. Thus, permanent changes occur in 5-HT and 5-HT_T after SCI, denervation 5-HT supersensitivity develops, and modulation of 5-HT attenuates pain-related behaviors. Insight gained by these studies may aid in the

understanding of dynamic 5-HT systems which will be useful in treating chronic central pain after SCI.

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Key Words: spinal cord injury; serotonin; transporter; denervation supersensitivity; pain.

INTRODUCTION

Spinal cord injury (SCI) results in a devastating loss of motor function below the level of the lesion as well as the development of chronic central 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 (23) which so severely compromises quality of life that suicide frequently ensues (16, 83). One mechanism involved in the origin and maintenance of central neuropathic pain may be the endogenous serotonin (5-HT) circuitry.

It is well known that descending serotonergic systems originate in brainstem raphe nuclei and terminate in the superficial dorsal and ventral horns of the spinal cord (12, 55, 87). There is considerable support for the role of 5-HT in modulating nociception (6, 10, 74) based on anatomical localization, behavioral effects of intrathecal drugs (45, 74, 91, 103), and inhibition of spinothalamic tract cells (STT) involved in pain transmission (98, 99, 100). 5-HT is known to play a facilitatory role in locomotor circuitry by increasing α -motoneuron excitability, modulating spinal central pattern generators (70, 71, 86), and improving locomotor behavior following spinal injury (50, 68). Relevant to SCI, deafferentation and denervation paradigms demonstrate diminished spinal levels of 5-HT acutely and ipsilateral to spinal lesion, with levels returning by 4 weeks after injury (62, 77, 108). These changes can be used as an indicator of injury severity (32) and provide a substrate for changes in somatosensory and locomotor behaviors. The hemisection model is valuable in this

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sense because of its selective surgical precision and sparing of contralateral 5-HT which can still exert an influence on the system unlike transection. As such, intrathecal replacement of 5-HT after hemisection should attenuate allodynia and hyperalgesia and improve locomotion via inhibition of aberrant neural circuits and facilitation of motor circuits, or both.

Another important molecule in the 5-HT system is the serotonin transporter (5-HT_T), which maintains 5-HT homeostasis by returning high extracellular synaptic concentrations to normal levels. Reports show that 5-HT_T is highly expressed in rat spinal cord and that its distribution parallels serotonergic innervation (28, 92) in dorsal horn laminae (I–IV), which are responsible for processing somatosensory information. Highest levels of 5-HT occur in laminae I and IIo (55), where the majority of primary nociceptive afferents project (10). As expected, 5-HT_T is thought to play a role in nociceptive processing since antinociceptive effects are produced by selective 5-HT reuptake inhibitors (34, 82) and tricyclic antidepressants (47, 78, 84, 94), which also inhibit serotonergic reuptake.

As of yet, no studies have examined changes in 5-HT and 5-HT_T at both cervical and lumbar enlargements at acute and chronic timepoints after unilateral hemisection injury and whether pharmacological manipulation can begin to provide insight into possible mechanisms of nociceptive modulation. The rationale to examine these segments is based on behavioral studies of chronically hemisected rats that demonstrate the development of mechanical allodynia and thermal hyperalgesia of the glabrous skin of 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 (22, 41). In the current report, we demonstrate significant changes in both spinal levels of 5-HT and 5-HT_T caudal and ipsilateral to a spinal hemisection. We also demonstrate attenuation of allodynia and hyperalgesia in both forelimbs and hindlimbs after SCI with 5-HT and fluvoxamine (a selective 5-HT reuptake inhibitor) and enhancement with methysergide (a 5-HT₁/5-HT₂ receptor antagonist), whereas the same drugs at the same doses had little effect in sham controls. We also demonstrate the development of denervation supersensitivity to 5-HT as a result of the spinal hemisection. Finally, we propose that continuous intrathecal delivery of 5-HT or a selective 5-HT reuptake inhibitor will attenuate abnormal somatosensory syndromes associated with chronic central pain after spinal cord injury.

METHODS

Subjects were male Sprague–Dawley rats, 100–125 g, obtained from Harlan Sprague–Dawley, Inc., housed with a 12-h:12-h light:dark cycle, acclimated and fed

ad libitum. All procedures 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

Animals (175–200g) were deeply anesthetized by intraperitoneal injection of sodium pentobarbital (40 mg/kg) and the surgical field was shaved and prepared with Betadine. A longitudinal incision was made exposing several segments and the left side of the spinal cord was laterally 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 vertebrae and counting two vertebral segments rostrally. The T13 spinal cord was laterally hemisected with a No. 11 scalpel blade without damage to the posterior spinal vessel or branches. Sham animals had only the dura cut without lesion to the spinal cord. Muscle and fascia were sutured 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 and the general health of the animals was carefully monitored. Rats were eating and drinking within 3 h of surgery. Weight loss was minimal, occurred acutely over the first postoperative 2 days, and was no greater than 5% of the normal body weight.

Tissue Analysis

Immunohistochemistry. At 3 ($n = 10$) and 28 ($n = 10$) days following spinal injury, both injured and sham ($n = 10$) animals were sacrificed and spinal cord sections processed for characterization of spatial and temporal changes in 5-HT and 5-HT_T staining density in laminae I–V. Animals were deeply anesthetized with sodium pentobarbital (60 mg/kg) and perfused intracardially with heparinized 0.9% saline followed by 4% cold buffered paraformaldehyde. After removal from the vertebral column tissue was postfixed for 24 h at 4°C in 30% sucrose. All 20- μ m cryostat-cut sections were processed concomitantly and uniform thickness was verified by through-focus techniques with an exclusion criteria set at values that did not fall within 1 standard deviation of this thickness. Every fifth section from both C8 and L5 segments was collected and incubated in 4% normal goat serum and 0.02% Triton X-100 in phosphate-buffered saline (PBS) for 20 min followed by rabbit polyclonal primary antibodies (5-

HT: Diasorin 1:5000; 5-HT_T: Diasorin 1:7000) in 0.02% Triton X-100 and PBS for 16 h. For 90 min at room temperature, goat antirabbit fluorescent secondary antibody (Texas Red, Molecular Probes, Eugene, OR) or biotinylated goat antirabbit IgG (Vector Laboratories, Burlingame, CA) followed by ABC-HRP Vector Elite kit reagents and DAB substrate with 0.01% H₂O₂ and NiCl₂ enhancement were added. Tissue was then cleared and mounted in either Permount (Fisher) or Anti-fade fluorescence mounting medium (Vector) for quantification and photomicroscopy. Controls included preabsorbed primary antibody and sections and absence of primary or secondary antibody.

Optical quantification of immunohistochemical product. After ensuring that staining was linearly related to both DAB and Texas Red secondary reporters, density of laminar 5-HT and 5-HT_T immunoreaction product was quantified utilizing optometric techniques. Analysis was performed bilaterally in the dorsal horn at both C8 and L5 using a Nikon light microscope coupled to a Pentium computer using Bioquant version 1.50 software. Sections ($n = 5$ /animal) from 10 animals at each time point were viewed with a 10X objective and images captured using a Spot Megapixel camera. Final projected image for analysis was 1000X that of the original spinal cord. Thus, pixels were included in the analysis that are not readily detectable in the low-magnification photodocumentation shown in the results. Integrated optical density area was confined and measured from individual laminae spanning the entire dorsal horn (laminae I–V). Density levels and distribution were quantified in hemisected rats at 3 and 28 days after injury and compared to data collected from shams. For all section, nonstaining gray matter was empirically chosen as background and staining was normalized to this signal intensity.

Western Blot Analysis

As a companion technique to immunohistochemistry to measure total 5-HT_T, animals were perfused with PBS and ipsilateral and contralateral spinal tissue was collected from C8 and L5 at 3 ($n = 5$) or 28 ($n = 5$) days postinjury, bisected into ipsilateral and contralateral sides, and homogenized in ice-cold phosphate-buffered saline containing 40 mM Tris–HCl (pH 7.5), 2% SDS, 2 mg/ml aprotinin, 2 mg/ml leupeptin, 2 mg/ml pepstatin-A, 2 mg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 1 mM EDTA. Homogenates from one segment were centrifuged at 10,000g for 10 min and the supernatant was collected and stored at –70°C. Protein concentrations of the homogenate were determined using the BCA Protein Assay Kit (Pierce, Rockford, IL). For gel loading, the homogenate was heated for 4 min at 95°C in an equal volume of sample buffer [100 mM Tris (pH 6.8) and 2% SDS, 2% 2-mercaptoethanol, 0.001% bromophenol

blue, and 20% glycerol] and then loaded onto a polyacrylamide gel in equal protein amounts (35 mg per lane). The stacking gel was 4% acrylamide, prepared in 0.13 M Tris (pH 6.8) and 0.1% SDS, and the separating gel was 10% acrylamide, prepared in 0.38 M Tris (pH 8.8) and 0.1% SDS. Samples were separated by electrophoresis in Tris–glycine buffer (25 mM Tris, 250 mM glycine, 0.1% SDS) at 200 V for approximately 25 min. Proteins were then transferred overnight (12 h) to a PVDF membrane at 30 V in transfer buffer containing 20% MeOH, 20 mM Tris, and 150 mM glycine (pH 8.0). Membranes were incubated for 1 h at room temperature in blocking buffer containing 5% nonfat powdered milk in TBS–Tween (20 mM Tris, 137 mM NaCl, and 0.1% Tween 20) and then washed for 10 min in TBS–Tween. Membranes were incubated overnight with primary antibodies (5-HT_T, 1:1000; Oncogene) in blocking buffer and Actin 1:10,000 (Upstate Biotechnology). After washing for 15 min with TBS–Tween, membranes were incubated in horseradish peroxidase-conjugated goat antirabbit IgG, diluted 1:10,000 in blocking buffer for 2 h, and then washed in TBS for 15 min. Peroxidase activity was detected using the Peirece SuperSignal West Femto Maximum Sensitivity Substrate kit and quantified using LabWorks software (UVP, Upland, CA). To control for variability in protein concentrations between samples, the ratio of signal intensities of 5-HT_T to actin were used to compare values and plotted as such.

5-HT ELISA

Tissue 5-HT content was quantitatively measured by a commercially available ELISA kit (ICN, Costa Mesa, CA). Tissue samples were collected from control animals ($n = 5$) and hemisected animals perfused with PBS at 3 ($n = 5$) and 28 days after injury ($n = 5$) and bisected into ipsilateral and contralateral sides. Samples were homogenized in ice-cold phosphate-buffered saline containing 40 mM Tris–HCl (pH 7.5), 2% SDS, 2 mg/ml aprotinin, 2 mg/ml leupeptin, 2 mg/ml pepstatin-A, 2 mg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 1 mM EDTA. Homogenates were centrifuged at 10,000g for 10 min and the supernatant was collected and stored at –70°C. Samples (20 μl) were then acylated (3% reagent) in 100 μl assay buffer. Standards, acylated control serum, and acylated samples (all 50 μl) were loaded into appropriate wells, and 50 μl each of serotonin-biotin and antiserum were added. The plate was sealed and incubated for 16 h at 4°C. After washing the wells with wash buffer, 150 μl of antibiotin AP was added and incubated for 120 min at room temperature. Following this step, the plate was washed and 200 μl substrate buffer was added and incubated for 60 min at room temperature until color development was achieved. The substrate reaction was stopped with 50 μl stop

solution and optical absorbance recorded at 450 nm with a microplate reader. The average of duplicated data was obtained and sampled concentrations were determined from the standard curve.

Intrathecal Drug Administration

In hemisected ($n = 10$) and sham ($n = 10$) animals at 28 days after injury, when nociceptive behavior was most robust in the hemisected group, a 32G intrathecal (i.t.) catheter (Micor Inc., Allison Park, PA) was filled with lactated PBS (pH 7.4), inserted through a slit in the atlantooccipital membrane, threaded caudally down the vertebral column to the T13 spinal subarachnoid space (as estimated by premeasured lengths), and sutured in place with an 8-mm length exiting the skin. The distal free end of the catheter was heat sealed to prevent infection. At sacrifice, we found no evidence of excessive fibrosis, fibrotic occlusion, or misplaced catheters. Animals were tested for predrug mechanical allodynia and thermal hyperalgesia as well as locomotor function. Immediately following these measures, different groups ($n = 10$ each) received either 5-HT (10, 21, or 63 μg ; RBI, Natick, MA) in a 10- μl solution equivalent to 5, 10, and 30 mM, the 5-HT₁/5-HT₂ receptor antagonist methysergide maleate (125 $\mu\text{g}/\text{kg}$ equivalent to 8.8 mM; RBI, Natick) or 5-HT reuptake inhibitor fluvoxamine maleate (75 $\mu\text{g}/\text{kg}$ equivalent to 5.7 mM, Tocris, Ballwin, MO). The doses were based on those previously published for 5-HT, wherein 100 μg is known to produce antinociception in normal rats but doses were adjusted downward due to known effects on mean arterial blood pressure (91) and possible denervation supersensitivity, which is known to occur after 5-HT depletion (45). Methysergide (1) and fluvoxamine (82) doses are published elsewhere. Drugs were administered in 10 μl normal PBS followed by a 15- μl PBS flush. Drug efficacy onset and offset points were determined empirically and behavioral values are reported from within the temporal window of drug efficacy. For 5-HT, at the 63- μg dose, the onset of antiallodynia began within 5 min of injection and lasted for 30 min. In the case of the multidose 5-HT-receiving animals, at least 24 h were allowed between each dose to allow for washout, at which time all rats were tested for recovery to predrug responses to eliminate possibility of desensitization or sensitization. Experiments show that when spinally hemisected rats are compared to hemisected rats with catheters in place, there are no significant differences in behavioral response profiles.

Control studies were performed to determine the distribution and penetration of i.t.-delivered 5-HT (63 μg in 10 μl) using immunolocalization techniques. Rats were perfused as described for immunohistochemistry and whole cords were examined by gross fluorescence microscopy followed by cryostat section analysis of the region immunopositive for 5-HT. In these animals

there was a significant signal that appeared over the dorsal third of one spinal segment, which penetrated to a depth of laminae I through V, bilaterally, just inferior to the termination of the i.t. delivery cannula (data not shown). No evidence of exogenous 5-HT-ir was measured in the dorsal, lateral, or ventral white matter or in laminae VI–X.

Locomotor Scoring

Post-SCI locomotor function was recorded by a blind observer for 28 days following hemisection injury using the Basso, Beattie, and Bresnahan (BBB) Locomotor Rating Scale (7) to ensure reliability of somatosensory testing and determine effects of i.t. drugs on open field locomotor function. After hemisection, animals demonstrating loss of locomotion in both hindlimbs, indicating bilateral corticospinal tract transection, were excluded from the study. With drug studies, since some doses might produce sedation which would significantly lower the values, scores were recorded for each dose and compound tested. Briefly, the BBB is a 21-point ordinal scale ranging from 0, which is no discernable hindlimb movement, to 21, which is defined as 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, and 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). As an additional control for drug effect, we observed each animal for 5 min during drug efficacy for the presence of tremors, which might be due to facilitation of motor circuits. We utilized a 4-point scale where 0 is no tremors, 1 is few isolated tremors, 2 is increased frequency of tremors but less than grade 3, and 3 is continuous tremors during the entire observation period (devised by Marion Murray, MCP/Hahnemann University). The agents in the present study produced no tremors or abnormal motor functions.

Mechanical Allodynia-von Frey

A blind observer performed behavioral tests to examine mechanical allodynia for both forelimbs and hindlimbs. Prior to testing, animals that were well handled for several days were environmentally acclimated to the clear Plexiglas cubicle testing apparatus (8 \times 8 \times 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. Drug trials began after 28 days

postinjury, at which time the responses to the mechanical stimuli have reached their maximum values.

Mechanical allodynia of the glabrous skin of the paw was quantified by measuring frequency of brisk paw withdrawals accompanied by active attention of the rat to the stimulus by head turning and stimulus attack, whole body posturing to avoid a repeated stimulus, and so on in response to normally innocuous low- and high-intensity mechanical stimuli (21). The inclusion of these complex behaviors excludes simple hyperreflexia, which is a segmental response (96, 101). Low-intensity stimuli were delivered by von Frey filaments (Stoelting, Wood Dale, IL) with bending forces of 4.78 mN and 9.96 mN, while high-intensity stimuli consisted of a von Frey filament with a bending force of 204.1 mN. In human perceptual terms, these mechanical forces are perceived as faint stimulus, light touch, and pressure, respectively.

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. Von Frey filaments were applied through the mesh to the ventral (plantar) surface of the skin of the paw for each limb. A single trial consisted of 10 applications of the filament, applied once every 3 to 4 s. The mean occurrence of paw withdrawals accompanied by supraspinal responses (above) in each of the trials was expressed as the number of responses of 10. Since no significant side-to-side limb differences were found, data from ipsilateral and contralateral limbs were collapsed into combined limb scores and presented as "forelimbs" and "hindlimbs" for ease of comparison. Data were analyzed and graphed as group data and compared to presurgical values and at 28 days and to various drug treatments for between group comparisons.

Thermal Hyperalgesia

Beginning at day 28 after injury, thermal hyperalgesia was measured by latency of paw withdrawal response to a heat lamp stimuli when accompanied by supraspinal response (8, 43). The Bennett-Hargreaves radiant heat stimulus was slightly modified to produce a slower-ramping heating phase to permit greater temporal separation of responses when comparing presurgical to postsurgical responses. Animals were placed in Plexiglas boxes on an elevated glass plate under which a radiant heat stimulus was applied by concentrating a beam of light onto the plantar surface of each paw. The light source is turned off automatically by a photocell upon limb lift, allowing measurement of time between start of light and paw withdrawal (withdrawal latency in seconds). Five minutes were allowed between each trial and three trials averaged for each limb, which were then combined. Since no significant side-to-side differences were found, data from ipsilateral and con-

tralateral limbs were collapsed into combined fore- or hindlimb scores for ease of comparison.

Statistical Analysis

All statistical tests were evaluated at the alpha 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 pairwise 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 drug treatment within each animal) or the two sample Student's *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 Jandel SigmaStat (version 1.0) and graphed using Jandel SigmaPlot (version 5.0) as mean \pm standard deviation (SD).

RESULTS

Immunohistochemistry and Quantification

The extent of the hemisection lesion, assessed histologically through sample sections (data not shown), was confined unilaterally and included the dorsal column, Lissauer's tract, lateral and ventral column systems, and gray matter. DAB and fluorescent staining properties such as saturation and signal intensity, as well as fading, were compared and controlled and the data collected from both procedures was found to be qualitatively similar and comparable. Photomicrographs of 5-HT immunofluorescence at cervical and lumbar enlargements taken from shams and 3 and 28 days following T13 spinal hemisection are shown in Fig. 1. In all sections, 5-HT immunoreactivity (ir) was highest in superficial dorsal horn laminae I-II. No significant changes were observed at C8 at any time points after injury for 5-HT. Reduction in L5 5-HT was observed acutely at 3 days postinjury on the ipsilateral side, which by 28 days remained decreased compared to sham animals. While staining returned somewhat by 28 days in the ipsilateral gray matter, densities did not achieve normal or contralateral levels. The density of 5-HT-ir product did not change in the contralateral L5 gray at any time points. Varicosities and increased 5-HT-ir in lamina X was observed in day 28 sections in the dorsal horn near the central canal (Fig. 2), and this dense bundle of fibers crossing the midline was detectable only in the lumbar enlargement of hemisected animals, not in the sham controls. Thus, providing documentation of contralateral serotonergic pathways,

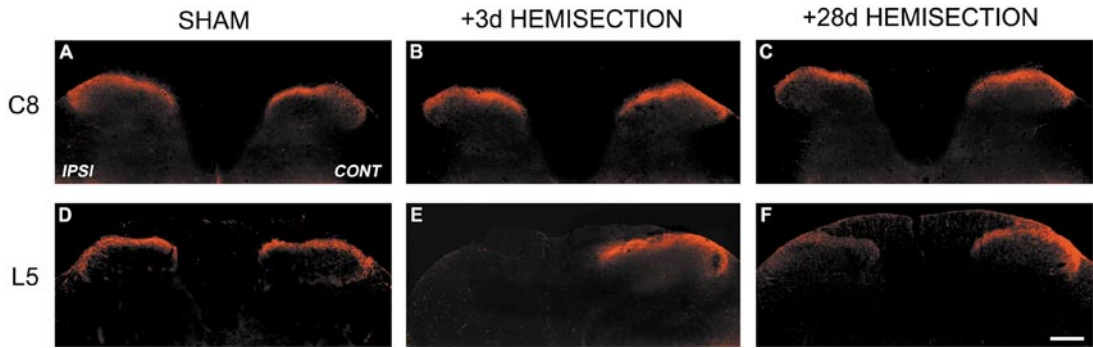


FIG. 1. Photomicrographs of 5-HT immunofluorescence from representative sections of C8 (A–C) and L5 (D–F) in sham-operated animals (first column) and day 3 or day 28 animals (middle and right columns, respectively) following T13 spinal hemisection. The ipsilateral dorsal horns are on left and the contralateral dorsal horns are on the right. Note that no changes in staining distribution or density were observed at the C8 level for any time point after injury; however, reductions in 5-HT staining intensity at L5 were observed acutely at 3 days postinjury ipsilaterally (E) compared to sham (D) or contralateral intraanimal dorsal horns (E). At 28 days after injury (F), the ipsilateral 5-HT staining intensity increased significantly compared to the day 3 expression level (compare E to F) but remained decreased compared to sham (D) or contralateral intraanimal dorsal horns. Calibration bar = 200 μm .

presumably the dorsal pathways, providing for the observed partial return of 5-HT in the dorsal horn (see below).

Laminar distribution of 5-HT_T staining was similar to that of 5-HT, although effects of hemisection were opposite. Photomicrographs of 5-HT_T-ir at cervical and lumbar enlargements taken from shams and 3 and 28 days following T13 spinal hemisection are shown in Fig. 3. 5-HT_T-ir was highest in the superficial dorsal horn (laminae I–II). No changes were observed at C8 at any time points after injury; however, L5 staining was increased ipsilateral to the hemisection at both 3 and 28 days postinjury. By 28 days, however, staining intensity had decreased compared to the day-3 time point but remained increased compared to control levels in the ipsilateral lumbar gray matter. Staining density did not return to normal or contralateral levels on this side.

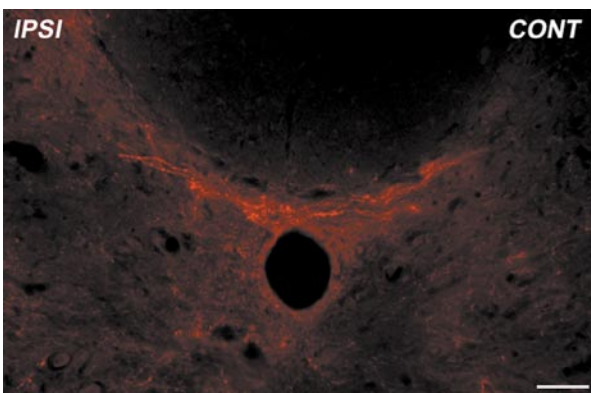


FIG. 2. Photomicrograph of 5-HT immunofluorescence taken at L5, 28 days following injury showing 5-HT-positive fibers and varicosities crossing the dorsal commissure in lamina X above the central canal. We hypothesize that the contralateral dorsal pathway is sprouting and serves as one of the sources of partial return of 5-HT in the dorsal horn. Calibration bar = 100 μm .

Quantification of ipsilateral and contralateral cervical and lumbar 5-HT-ir in sham rat spinal cord and at 3 and 28 days following T13 hemisection injury is shown in Fig. 4. In C8, ipsi- and contralateral 5-HT-immunoreactive units were not significantly different at both 3 and 28 days posthemisection compared to shams. By 3 days posthemisection, a significant ($P < 0.05$) reduction in 5-HT signal intensity (arbitrary units) was observed in the ipsilateral lumbar enlargement, in laminae I (20 ± 5) and II (42 ± 7), but not on the contralateral side, 162 ± 7 and 158 ± 9 , respectively. At 28 days, ipsilateral 5-HT-ir remained significantly decreased in laminae I (101 ± 8) and II (107 ± 7) when compared to sham animals or the contralateral side. Ipsilateral and contralateral 5-HT_T-ir was not significantly different in the cervical enlargement from shams at any time points (Fig. 4). By 3 days posthemisection, a significant increase in 5-HT_T signal intensity was observed in the ipsilateral lumbar enlargement in laminae I (168 ± 6) and II (143 ± 5), but not on the contralateral side, compared to shams, 133 ± 4 and 114 ± 3 , respectively. At day 28, ipsilateral 5-HT_T-ir remained significantly increased in laminae I (146 ± 6) and II (139 ± 7). Contralateral staining remained unchanged at all time points.

5-HT_T Western Blot Analysis

Western blot analysis of 5-HT_T is shown in Fig. 5. Samples taken from C8 spinal segments expressed basal levels of 5-HT_T, which after T13 hemisection remained unchanged at either 3 or 28 days relative to sham. In contrast, ipsilaterally at L5 3 days after injury, significant ($P < 0.05$) up-regulation of 5-HT_T was observed when compared to sham-operated animals and/or the contralateral side, up to 1.10 ± 0.10 (% actin, arbitrary units) from 0.59 ± 0.05 . At day 28 this ipsilateral up-regulation was decreased compared to

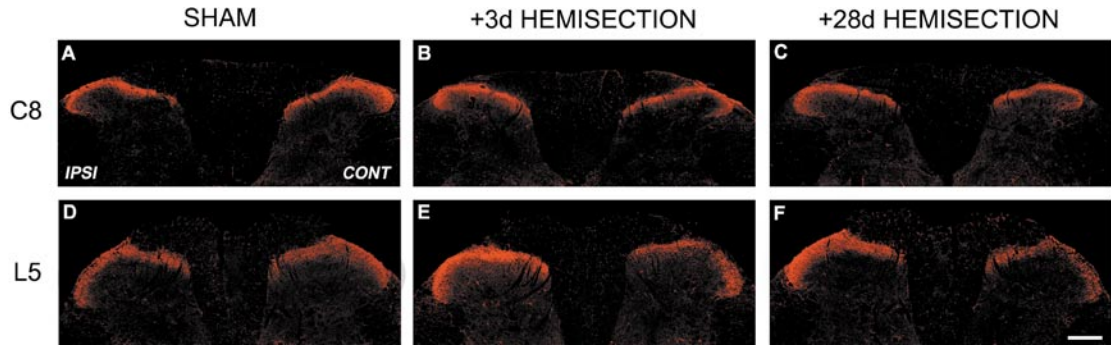


FIG. 3. Photomicrographs of 5-HT_T immunofluorescence photomicrographs of representative sections of C8 (A–C) and L5 (D–F) in sham-operated animals (first column) and day 3 or day 28 animals (middle and right columns, respectively) following T13 spinal hemisection. The ipsilateral dorsal horns are on left and the contralateral dorsal horns are on the right. No changes in staining distribution or density were observed at C8 at any time point after injury; however, increased 5-HT_T staining intensity at L5 was observed acutely at 3 days postinjury ipsilaterally (E) compared to sham (D) or contralateral intraanimal dorsal horns (E). The increased staining intensity observed ipsilaterally remained increased at 28 days after injury compared to sham (D) or contralateral intraanimal dorsal horns but decreased compared to day 3 animals. Calibration bar = 200 μ m.

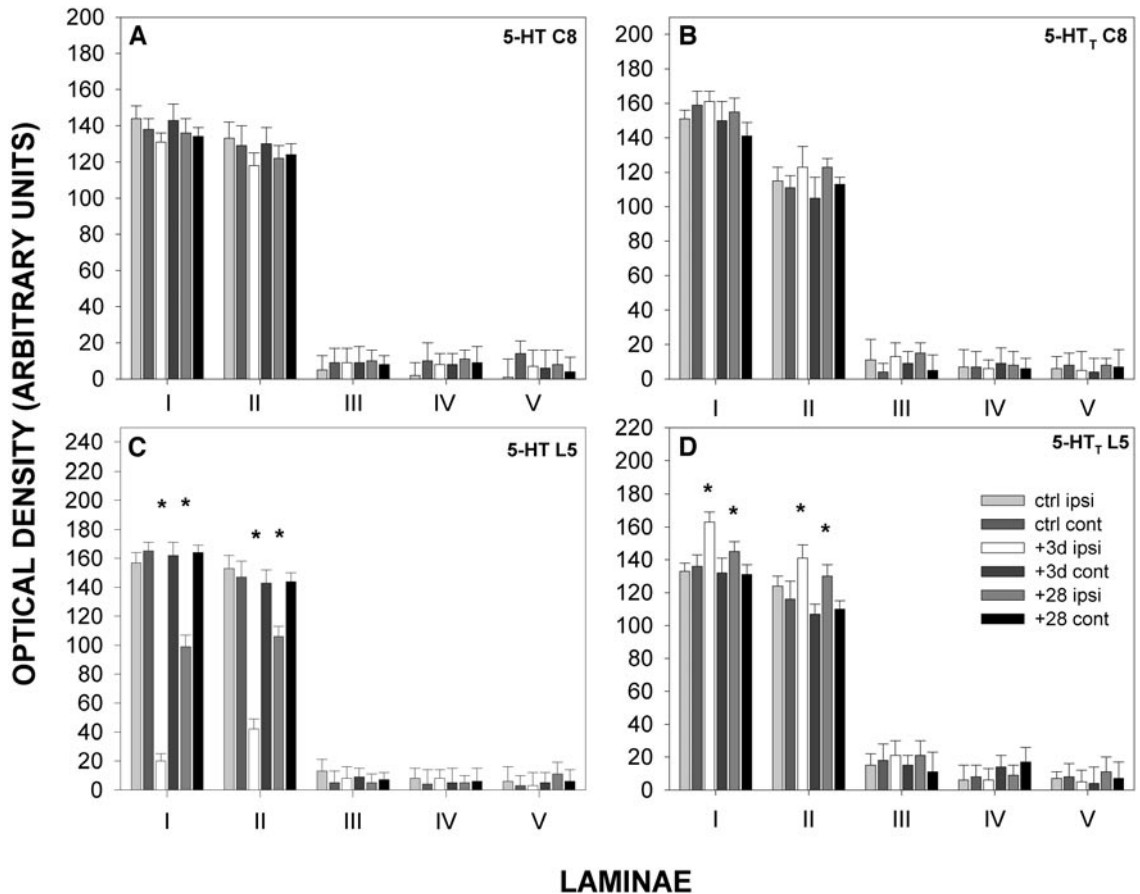


FIG. 4. Optical intensity values of the mean \pm SD measured from ipsilateral (ipsi) and contralateral (cont) dorsal horns at C8 and L5 for 5-HT (A and C) and 5-HT_T (B and D) immunoreactivity in sections from sham control (ctrl) spinal cords and sections from spinal cords at 3 (+3d) and 28 (+28) days following T13 spinal hemisection injury. A significant ($P < 0.05$) reduction in ipsilateral 5-HT signal intensity compared to sham controls was observed acutely at 3 days in L5 laminae I and II but not contralaterally or in sections from C8. By 28 days, 5-HT signal intensity was partially restored but remained significantly decreased in laminae I and II compared to sham control values ($P < 0.05$) and to the contralateral dorsal horns. The intensity of 5-HT_T reaction product was not significantly changed in C8 but in L5 the intensity of reaction product was significantly increased ipsilateral to the hemisection at both 3 and 28 days postinjury in laminae I and II compared to sham controls ($P < 0.05$).

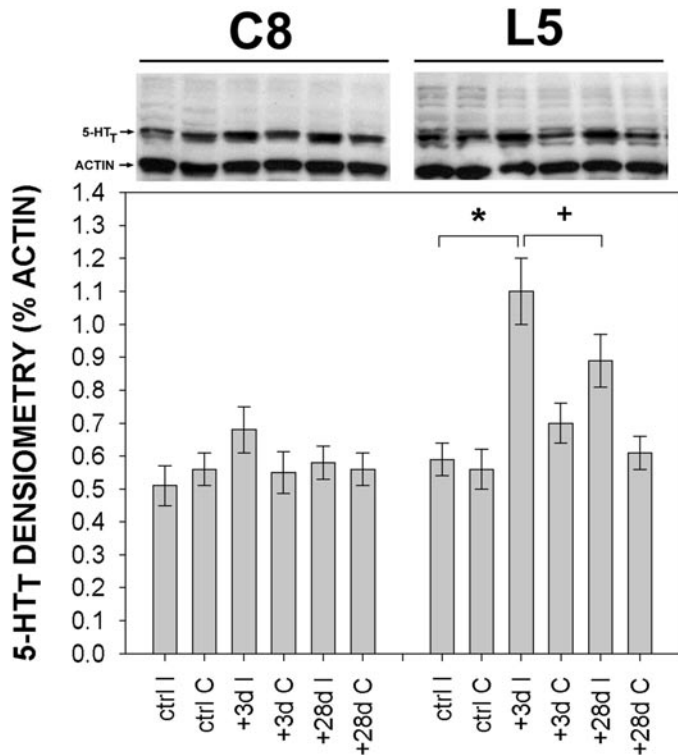


FIG. 5. Western blot analysis and densitometric quantification of serotonin transporter (5-HT_T) bands from C8 and L5 samples (representative blot shown at the top) taken from the ipsilateral (I) and the contralateral (C) sides of spinal cords collected from sham control animals (ctrl) and animals 3 (+3d) and 28 (+28d) days after hemisection. The densities are shown as mean \pm SD in units of the percentage of actin density, which served as the control protein. No significant changes were observed at C8 under any conditions, but at L5 significant ($*P < 0.05$) 5-HT_T up-regulation was observed on the ipsilateral side at 3 and 28 days after spinal hemisection injury.

day 3 values (to 0.89 ± 0.08) but remained significantly increased relative to shams or the contralateral side. These data extend and confirm histological quantification. Since 5-HT is a single amino acid and not a protein or peptide, Western blot analysis is not the method of choice for quantitative analysis. Thus we used ELISA analysis for 5-HT quantification.

5-HT ELISA

Tissue levels of 5-HT are shown in Fig. 6. At C8 there were no significant changes in mean concentrations of 5-HT at any time point or in comparison to control levels, 42.3 ± 3.17 and 41.0 ± 2.51 (ng of 5-HT/mg of wet weight of tissue) for ipsilateral and contralateral sides, respectively. At L5, on the ipsilateral side, significant ($P < 0.05$) decreases were observed in 5-HT tissue content at 3 days postinjury, decreasing to 33.4 ± 3.40 from 41.2 ± 3.57 ng/mg. Contralateral levels were unchanged, which remained at 40.2 ± 2.65 . By 28 days, levels remained significantly decreased on the ipsilateral side, at 34.2 ± 3.28 , but not on the contralateral side, 39.6 ± 2.24 ng/mg.

Intrathecal Drug Administration and Behavioral Testing

Locomotor function. Immediately upon emerging from anesthesia, hemisected animals displayed loss of ipsilateral hindlimb function. Animals demonstrating deficits in contralateral limbs or displaying signs of autophagia were excluded from the study. Over the course of 4 weeks, rats gradually regained use of their ipsilateral hindlimb, the progress of which can be measured using the BBB scale (Fig. 7). 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, knee, and finally the ankle joints. By the time of drug testing (4 weeks), the mean \pm SD BBB score was increased significantly ($P < 0.05$) to 14.3 ± 1.02 and did not change during drug treatments. Forelimb and contralateral hindlimb motor scores were unaffected by the T13 hemisection, which remained at 21 throughout the study. BBB scores were unchanged throughout the various doses tested up to the maximum of 5-HT, (63 μ g), methysergide (125 μ g/kg), or fluvoxamine (75 μ g/kg).

It might be thought that since ipsilateral hindlimb BBB scores had not returned to normal values, that responses to mechanical and thermal stimuli would be affected. This is not the case, however, since at the time of mechanical and thermal testing the ipsilateral hindlimb had recovered significantly as shown by the BBB scale. Although the BBB is widely used in the SCI community, its nonlinear nature may be misleading. In

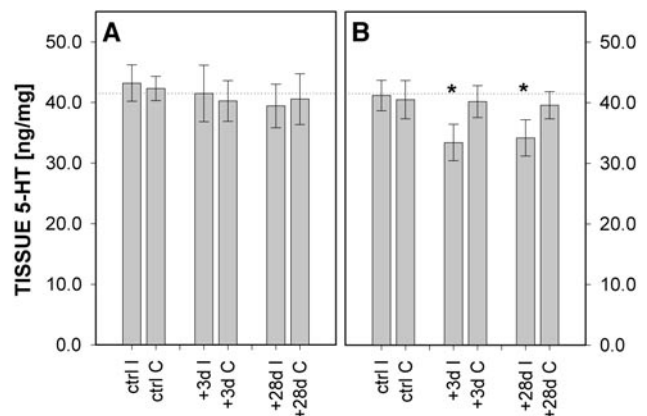


FIG. 6. ELISA analysis of serotonin (5-HT) levels in spinal tissue from C8 (A) and L5 (B) samples taken from the ipsilateral (I) and the contralateral (C) sides of spinal cords collected from sham control animals (ctrl) and animals 3 (+3d) and 28 (+28d) days after spinal hemisection. Data are shown as mean \pm SD of nanograms per milliliter of supernatant. Note that no changes are evident in tissue from the C8 spinal segment. By contrast, in the L5 segment, significant ($*P < 0.05$) decreases in spinal 5-HT were evident on the ipsilateral side, compared to sham control values, as soon as 3 days postinjury. The significantly decreased 5-HT levels ($P < 0.05$) ipsilateral and caudal to the T13 spinal hemisection persisted for 28 days.

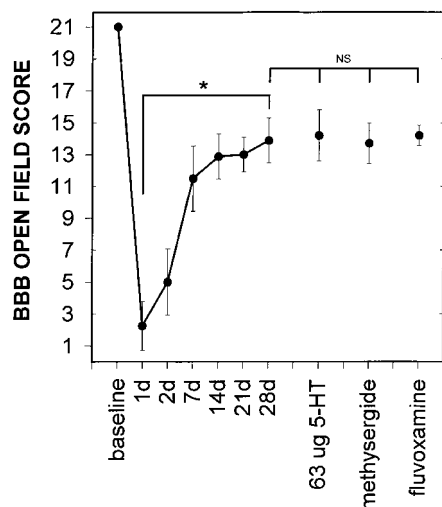


FIG. 7. Mean \pm SD BBB open field locomotor rating scale scores (6) of the ipsilateral hindlimbs in the animals used in the behavioral portion of this study after spinal hemisection at T13, plotted in days after injury. Both forelimbs and the contralateral hindlimb scored 21 throughout the testing period. Twenty-one represents normal locomotion, and zero represents no observable movement. On postsurgical day 1, animals scored a 2.25 ± 1.52 and by day 28 motor scores had increased significantly ($*P < 0.05$) to 14.3 ± 1.00 , with little significant variation for the remainder of the study. These results are consistent with recovery of locomotion in other hemisection studies (22, 23, 41). The important observations in these data are that the group of animals demonstrated no significant alterations in locomotor function while under the influence of the highest dose of 5-HT (63 μg) or at the doses of methysergide (125 $\mu\text{g}/\text{kg}$) or fluvoxamine (75 $\mu\text{g}/\text{kg}$) used in the current study.

these experiments, a score of 14 of 21 does not indicate 66% recovery but lack of fine control of digits, with consistent weight supported steps and consistent FL–HL coordination, with predominant paw position rotated at initial contact and liftoff.

Mechanical stimuli. Mean \pm SD number of paw withdrawals to von Frey filaments at 4 weeks postinjury for the combined ipsilateral and contralateral limbs are shown in Fig. 8. The sham control group had no significant response to the compounds at the doses used in this study (Figs. 8A–C). For the spinal hemisection group, compared to preinjury time points, values were significantly ($P < 0.05$) increased for both fore- and hindlimbs at all time points (Figs. 8D–F). For forelimbs, presurgical animals exhibited 0.38 ± 0.12 withdrawals to a 4.78 mN mechanical stimulus and 0.88 ± 0.18 withdrawals to a 9.96 mN mechanical stimulus, which following spinal hemisection significantly ($P < 0.05$) increased, reaching a plateau of 4.20 ± 0.45 and 4.63 ± 0.80 withdrawals for the 4.78- and 9.96-mN hairs, respectively. In the high-intensity 204.1-mN group, number of forelimb withdrawals significantly increased from 3.71 ± 0.52 to 7.75 ± 0.49 . Similarly, hindlimb responses were significantly increased from initial values of 0.29 ± 0.11 to 3.52 ± 0.40

for postsurgical values with the 4.78 mN filament and from 0.79 ± 0.16 to 4.20 ± 0.42 with the 9.96-mN stimulus. Hindlimb withdrawals significantly increased from 2.51 ± 0.20 to 6.25 ± 0.96 with the 204.1-mN stimulus.

Rats were then tested after i.t. injections of incrementing (vehicle and 10-, 21-, or 63- μg) doses of 5-HT (Fig. 8). Number of paw withdrawals of both fore- and hindlimbs significantly decreased as 5-HT doses increased. These 5-HT responses were not significantly different from presurgical values in some cases. Using correlation statistics to compare paw withdrawal to increasing 5-HT doses, 5-HT produced a significant ($P < 0.05$) forelimb correlation coefficient (CC) of -0.588 ($P = 0.00641$) for 4.79-mN filaments and -0.0471 ($P = 0.0483$) for 9.96-mN filaments. Similarly, significant hindlimb CC's were -0.468 ($P = 0.0375$) and -0.712 ($P = 0.000428$) for 4.48- and 9.96-mN filaments, respectively. Correlation coefficients for the 204.1-mN filament also demonstrated a statistically significant dose-related decrease in forelimbs (CC = -0.582 , $P = 0.007$) and hindlimb (CC = -0.544 , $P = 0.013$) withdrawals to increasing i.t. doses of 5-HT. At washout 90 min following 63 μg injection, there was no behavioral evidence of permanent desensitization or sensitization, as fore- and hindlimbs demonstrated nociceptive behaviors after this period for all intensity fibers that were consistent and not significantly different from presurgical values. Thus, there is a dose-related attenuation of paw withdrawal response to mechanical stimuli, with 63 μg being most robust.

Intrathecal methysergide and fluvoxamine resulted in significantly enhanced or attenuated number of paw withdrawals, respectively (Fig. 8). Methysergide increased paw withdrawal frequency at all intensity filaments, with significance achieved in 4.79 mN in both fore- and hindlimbs, up to 4.99 ± 0.21 and 5.23 ± 0.22 from lower day-28 time points. Similar effects were seen in forelimbs at 9.96 mN with frequency increasing to 5.45 ± 0.31 , and for both fore- and hindlimbs at 204.1 mN, increasing to 9.68 ± 0.62 and 9.98 ± 1.21 , respectively. Fluvoxamine resulted in significantly decreased withdrawals in forelimbs at 4.79 mN to 3.67 ± 0.22 , fore- and hindlimbs at 9.96 mN to 2.49 ± 0.28 and 2.88 ± 0.29 , and forelimbs at 204.1 mN to 5.25 ± 0.96 . Vehicle injections did not significantly alter the postsurgical values.

Thermal Stimuli

In the sham control group, both forelimb and hindlimb thermal behavior was not significantly different from baseline behavior (Fig. 9A). For the spinal hemisection group, beginning at 28 days, mean \pm SD paw withdrawal latencies to thermal stimulation were significantly decreased following spinal hemisection (Fig. 9B). From control (baseline) withdrawal latencies of

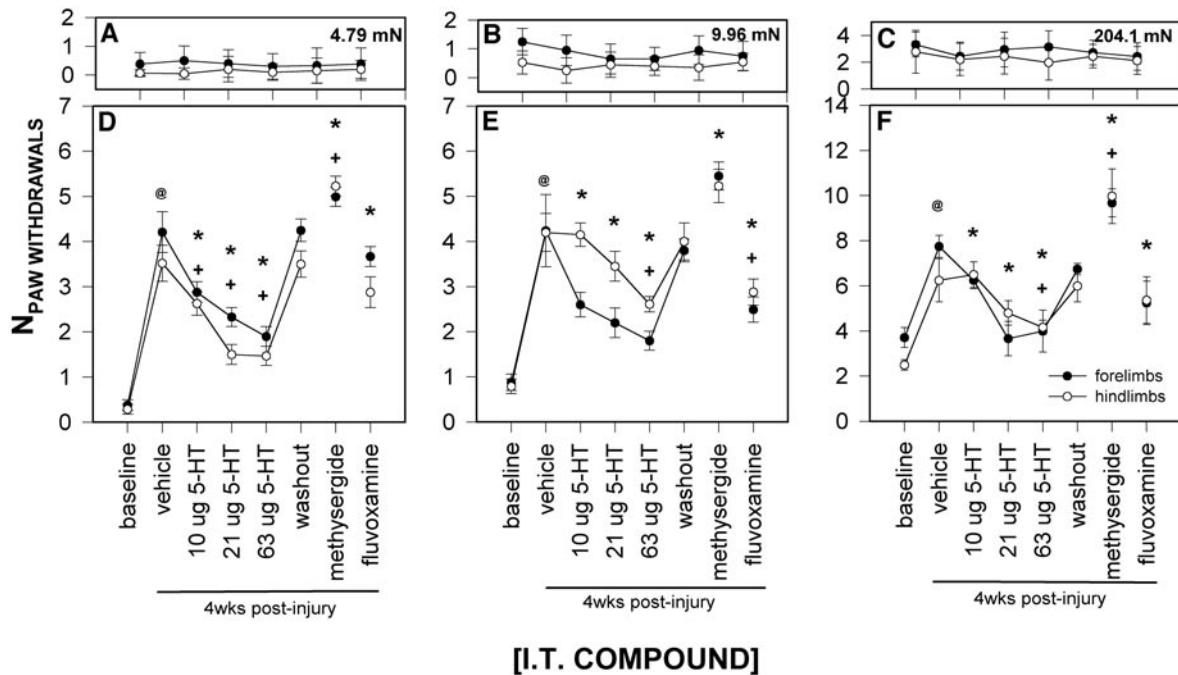


FIG. 8. Combined left and right forelimb (filled circles) and left and right hindlimb (open circles) number of paw withdrawal responses following sham surgery (A, B, and C) or spinal hemisection (D, E, and F) and intrathecal administration of different doses of 5-HT (10, 21, 63 μ g), methysergide (125 μ g/kg), or fluvoxamine (75 μ g/kg) plotted as responses after application of von Frey filaments with bending forces of 4.78 mN (A and D), 9.96 mN (B and E), and 204.1 mN (C and F), to the glabrous skin. All drug doses tested began on days 28 after injury when animals had developed mechanical allodynia ($@P < 0.05$), when both locomotor and somatosensory responses are stable in the hemisection model. Values are graphed as mean \pm SD number of paw withdrawals of 10 tests for the animals in each group. 5-HT doses are connected not to show temporal order but to demonstrate dose relatedness of the response. The postdrug response following 63 μ g 5-HT is shown, although other doses and drugs demonstrate similar returns to postsurgical (vehicle) responses. Asterisks (forelimbs) and plus symbols (hindlimbs) represent statistically significant ($*+P < 0.05$) differences compared to vehicle-alone values. There were no significant responses to the drugs tested in sham control animals (A, B, and C).

13.2 \pm 0.17 (s) and 26.1 \pm 0.50 for fore- and hindlimbs, rats demonstrated significantly decreased ($P < 0.05$) latencies of 8.85 \pm 1.13, and 21.2 \pm 2.16, respectively. Decreases in paw withdrawal latency were accompanied by whole-body changes such as attacks to the stimulus, writhing, and movement of the body away from the stimulus and paw licks.

Intrathecal 5-HT resulted in significant increases in paw withdrawal latencies (Fig. 9). In both fore- and hindlimbs, 5-HT (63 μ g) resulted in a significant ($P < 0.05$) increase in paw withdrawal latencies compared to vehicle at 11.1 \pm 0.43 and 25.6 \pm 1.42 s respectively. At 90 min following a 63- μ g injection, there was no permanent desensitization or sensitization as animals exhibited withdrawal latencies of 8.33 \pm 0.33 and 21.6 \pm 2.22, for fore- and hindlimbs respectively. Thus, the postdrug thermal latencies following a 63- μ g dose demonstrated a return to values similar to the vehicle alone responses. Intrathecal methysergide resulted in significantly decreased withdrawal latencies in both fore- and hindlimbs, 6.88 \pm 0.90 and 18.0 \pm 0.90 respectively, while fluvoxamine had no significant effects at the dose tested. Although some studies noted desensitization after repeated 5-HT administration (200 μ g

given every 5 min) (35), there were no data from the current study to support cumulative, desensitization effects of multiple low doses of 5-HT with the doses and dosing regimen used in this study.

DISCUSSION

The present study confirms that rats spontaneously recover considerable ipsilateral hindlimb locomotor function following spinal cord hemisection (23, 77), as well as develop behavior consistent with mechanical allodynia and thermal hyperalgesia in both forelimbs and hindlimbs (22). We also report that hemisection results in no changes in 5-HT or 5-HT_T expression in the C8 segment of the cervical enlargement, but that significant ipsilateral decreases in 5-HT expression occurred in L5 which recover partially and significant ipsilateral increases in lumbar 5-HT_T expression occurred as determined by immunohistochemical, Western blot, and ELISA analyses. Additionally, we report that i.t. administration of 5-HT significantly attenuates mechanical allodynia and thermal hyperalgesia in both fore- and hindlimbs and that methysergide (a 5-HT₁/5-HT₂ receptor antagonist) increases mechani-

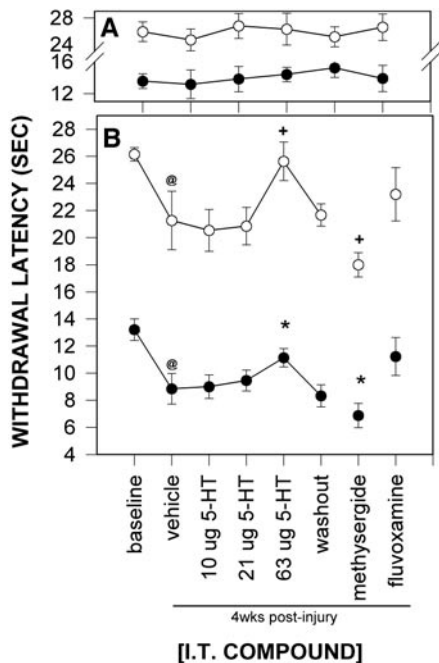


FIG. 9. Combined left and right forelimb (solid circles) and left and right hindlimb (open circles) paw withdrawal latencies, in seconds (s), to thermal stimuli following sham surgery (A) and spinal hemisection (B) and intrathecal administration of different doses of 5-HT (10, 21, and 63 µg), methysergide (125 µg/kg), and fluvoxamine (75 µg/kg). All drug doses tested began on days 28 after injury when rats demonstrated thermal hyperalgesia ($P < 0.05$) and when both locomotor and somatosensory responses are stable in the hemisection model. Values are graphed as mean \pm SD paw withdrawal latency. 5-HT dosages are connected not to show temporal order but for ease in demonstrating any dose relatedness of the response, which in this case is not related. The postdrug response following 63 µg 5-HT is shown, although other doses and drugs demonstrate similar returns to postsurgical (vehicle) responses. Asterisks (forelimbs) and plus symbols (hindlimbs) represent statistically significant ($*+P < 0.05$) differences compared to vehicle-alone values. There were no significant responses to the drugs tested in sham control animals (A).

cal allodynia and thermal hyperalgesia, while fluvoxamine (5-HT_T inhibitor) decreases only mechanical allodynia. No effect on locomotor function was found with the doses of 5-HT, methysergide or fluvoxamine used in these studies.

With respect to the changes in expression of 5-HT and 5-HT_T, it is notable that the dorsal horn caudal and ipsilateral to spinal hemisection exhibits decreased 5-HT in laminae I and II, but increased 5-HT_T levels. This is particularly interesting since the transporter is known to be associated with 5-HT terminals and other investigators have shown decreased expression of transporter after chemical lesions of serotonergic terminals in neonatal rat (64, 65). We propose that due to the loss of local 5-HT, 5-HT_T in remaining neural cells is up-regulated. The up-regulation of 5-HT_T decreases with time as 5-HT returns partially, but does not reach presurgical levels. We suspect that 5-HT_T

down-regulation occurs in response to partially restored 5-HT, a mechanism supported by data demonstrating that acute depletion of 5-HT induces increases in 5-HT_T mRNA (46, 106) and relationship between receptor and transporter (66). The difference in 5-HT_T expression in our study compared to the neonatal studies may be methodological (agonist binding versus immunocytochemistry, Western blot, and ELISA), age at time of injury (neonate versus adult) and type of injury (chemical and complete lesion versus incomplete surgical lesion in which 5-HT projections regenerate). Additionally, the cellular localization of 5-HT_T is not known and could reside on presynaptic or postsynaptic neurons and/or glia.

Our plastic changes in 5-HT staining intensity in laminae I and IIo, where the nociceptive-mediating (102) 5-HT_{1A} receptor subtype resides (56), are similar to results reported by others after rhizotomy (62), capsaicin treatment (57), or hemisection (77). Our immunocytochemistry results were confirmed by alternate techniques in this study, Western blot analysis (for 5-HT_T) and ELISA (for 5-HT), but it must be remembered that with Western and ELISA techniques, both dorsal and ventral hemicords were collected in unilateral samples which might skew the results as immunohistochemical data considered only dorsal horn staining intensity. That is, both 5-HT and 5-HT_T are localized to superficial laminae I and II cells within the dorsal horn, in the ventral horn, and most likely in the intermediolateral cell column. The regional differences may contribute to differences observed in these assays. Our report of caudal, ipsilateral reductions of 5-HT in the dorsal horn immediately after injury with increased expression over time that correlated with improvement in functional neurological outcome is consistent with work by others in spinal injury models (13, 32, 62, 77, 108), including reports of partial return of 5-HT in the dorsal horn after hemisection (77). Another group, however, reported that 5-HT does not return to the dorsal horn on the ipsilateral side 5 weeks after hemisection unless oxysterol was administered intrathecally caudal to the hemisection (37). The reason for this discrepancy is not clear but may be due to genetic differences in 5-HT pathways, regenerative responses, and so on. The mechanisms proposed for the observed return of 5-HT over time are regenerative sprouting of ipsilateral and/or contralateral 5-HT pathways (20, 77, 87, 109, 110), increased 5-HT synthesis and metabolism within spared neuronal elements remaining after injury (40), or both.

It is well accepted that the descending serotonergic system is composed of three general pathways: the dorsal, intermediate, and ventral pathways. The dorsal pathway consists of neurons originating mainly in the rostral raphe magnus, the reticularis gigantocellularis pars alpha, the paragigantocellularis lateralis, and the raphe magnus proper nuclei, the projections of which

travel in the dorsal lateral fasciculus and terminate mainly ipsilaterally but to some extent cross at the level of termination in the superficial, intermediate, and deep dorsal horns. The intermediate pathway consists of neurons originating mainly in the arcuate nucleus but also from raphe obscurus and pallidus nuclei, the projections of which travel in the mediolateral region of the lateral funiculus and terminate both ipsilaterally and some contralaterally in the intermediolateral cell column. Finally, the ventral pathway consists of neurons originating in the raphe obscurus and raphe pallidus nuclei, the projections of which travel in the ventral and ventrolateral funiculi and terminate both ipsilaterally and contralaterally in the area of somatic motor neurons in the ventral horn (87). The time course of reemergence of 5-HT after spinal injury corresponds temporally with recovery of locomotion (44, 77). We propose that the persistence of painlike behavior reflects the inadequate return of 5-HT levels as well as changes in 5-HT transporter and receptor populations. Another possibility is that there are differences in the numbers of crossed projections of the dorsal and ventral serotonergic pathways such that there are greater crossed projections in the ventral pathway, allowing for more rapid 5-HT restoration in the ventral horn, compared to the dorsal pathway projections, in which crossed projections may be considerably less (37, 87). This anatomic arrangement would account for the persistent nociceptive behavior despite partial concomitant recovery of locomotion after spinal hemisection.

Intrathecal delivered 5-HT is generally thought to affect both nociceptive and locomotor behavior. However, previous studies produced conflicting results with respect to the antinociceptive responses of exogenous 5-HT. Some studies demonstrated that i.t. 5-HT in normal rats produced antinociceptive responses in the formalin test (17), dose-dependant increases in hot plate and tail flick latencies (17, 27, 79, 91, 103), increased vocalization thresholds during shock (11), and decreased tail- and monosynaptic withdrawal reflexes (24, 26) by both facilitation and inhibition (25). However, other studies demonstrated weak (52) or no thermal alterations (60, 105) or the requirement of increased concentrations of 100- to 1000-fold higher for effectiveness (4). Our data corroborate some of these studies in that 5-HT reuptake inhibition with fluvoxamine attenuates mechanical allodynia; however, 5-HT (63 μg) attenuates both mechanical and thermal responses. One possible explanation for the discrepancy in our data is that the concentration of fluvoxamine used in the present study was not sufficiently high enough to influence thermal nociception. Another is that fluvoxamine could elevate extracellular endogenous 5-HT to levels higher than 63 μg i.t. 5-HT, resulting in subtle facilitation of motor circuits not revealed by our locomotor assessment and thus any analgesic

effect would be masked. This might decrease paw withdrawal reaction times and subsequently mask significant antinociceptive action (longer paw withdrawal latencies) in thermal testing paradigms. Additionally, there may be greater relative adrenergic modulation of thermal nociceptive circuitry, compared to serotonergic modulation, that would lessen the influence of 5-HT, particularly on 5-HT_{1A} receptors (60). Finally, differences in the cellular level of the distribution of 5-HT_T on different neuronal circuits (thermal vs mechanical) could account for the behavioral data. All of these mechanisms are testable by direct measurements of extracellular 5-HT by microdialysis, cellular colocalization studies and neuropharmacological/electrophysiological analyses of the neural circuits involved.

The doses used in the present study to attenuate nociceptive responses after spinal hemisection (10 to 63 μg) were approximately half or less than those reported to produce antinociception in normal rats (100 to 200 μg) (45, 91, 103). This is not surprising since it is known that interruption of the innervation of neural tissue can enhance the responsiveness of that tissue to the action of agonists (18). For example, there are other studies in which lesioning of descending 5-HT pathways induces denervation supersensitivity to 5-HT, which alters the antinociceptive effect of 5-HT receptor stimulation (30, 31, 45, 64, 93) similar to what we observed. After hemisection, our animals were exquisitely more sensitive than controls to i.t. 5-HT and become supersensitive to previously ineffective drug concentrations. This denervation supersensitivity is manifested as a shift to the left of the agonist dose-response curve shown by others (45) and explains the tendency of injured animals to respond to lower concentrations of 5-HT and related pharmacological agents.

Monoamines most certainly play a role in hemisection-induced denervation changes in locomotor function. Local spinal central pattern generator (CPG) circuitry responsible for rhythmic control of limb movements is modulated by descending monoaminergic inputs (2, 3, 70, 80). Two modulator-specific pathways, noradrenergic fibers from the locus coeruleus and serotonergic fibers from the caudal raphe nuclei, have been shown to be involved (35). First, with regard to serotonergic fibers terminating in the ventral horn, as stated above, there exist different pathways and different proportions of ipsi- and contralateral projections when compared to the dorsal horn (12, 36, 67, 87). Electrolytic and chemical (5,7-dihydroxytryptamine) lesions of the midline raphe nuclei, which deplete 5-HT systems, result in permanent decreases in locomotor activity (71, 72), and as expected, 5-HTP injections into the ventral horn of the spinal cord facilitate spontaneous rhythmic locomotor activity in awake animals (95). The 5-HT_{1C} and 5-HT₂ receptor antagonist mianserin both improves (73) and adversely affects (75) locomotor

function after injury. This effect is suspected to involve dynamic control of CPG circuitry. Serotonergic neurons display a dramatic elevation of neural activity during the expression of repetitive CPG-mediated motor outputs, facilitating motor output and simultaneously suppressing sensory processing (48, 49).

The location of the CPG has for many years been debated, and only recently has its anatomical location been clarified. In cats, chronic, low thoracic lesions produce transient locomotor deficits that partially recover over time (14). Following spinal transection, transplantation of embryonic raphe cells results in recovery of rhythmic locomotor function and increased responsiveness to reflex tests (33, 88) only at segments L1-L2 (68) where most CPG circuitry resides in the ventral horn (19, 51). The effectiveness of 5-HT is hypothesized to be on excitability of host neurons through increases in amplitude of monosynaptic reflexes in central pattern generator circuitry (97). Studies in neonatal rat indicate that agonists for 5-HT directly depolarize neurons (9) and that 5-HT can indirectly influence motoneuron excitability by attenuation of both excitatory and inhibitory synaptic inputs (53, 105). In the present study, the relatively low i.t. 5-HT doses used produced no detectable changes in motor function such as tremors or open field locomotor deficits in either sham or hemisectioned groups.

We did observe vocalization and complex behavioral responses to nociceptive stimulation below the level of lesion, which may seem paradoxical since this region is generally thought to be insensate; however, there are anatomical substrates for supraspinal transmission of peripherally evoked nociceptive information caudal to the lesion, both ipsilaterally and contralaterally (61, 69, 100). The contralaterally affected area following STT tractotomy can be observed by the uninterrupted spinocervicothalamic tract, which carries nociceptive information (29), and by the population of STT cells (18%) that project ipsilaterally (15). In addition, the spinoreticular tract mediates somatosensation for both sides (81), and short-fiber multisegmental propriospinal pathways are able to relay similar information from one side of the cord to another (5). Furthermore, in the cat 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 (89, 90) which could contribute in part, to nociceptive changes in other regions distant and above the lesion, such as forelimb responses. Thus, alterations in sensory processing are not necessarily dependent on the sidedness of SCI, but on the tracts and cellular processes altered.

With regard to the effectiveness of a T13 i.t.-delivered compound effecting forelimbs, there is evidence in animal models and in patients that hyperexcitability occurs in the cord immediately above and below the level of lesion (23, 54, 58, 104). Hyperexcitability in one

region of the circuit would effect the entire circuit, i.e., both fore- and hindlimbs, particularly in quadrupedal animals, where locomotor circuits such as flexor/crossed extensor reflex exist (39, 85). Consequently, i.t.-delivered agents, with limited diffusion capabilities (59, 79, present study) targeted at or near the hemisection site would be effective in modulating forelimb and hindlimb sensitivity through reduction in local abnormal circuit hyperexcitability, in the case of inhibition, that would in turn decrease the gain of several somatosensory pathways, including nociceptive pathways (41). Electrophysiological studies are currently in progress to examine these proposed circuit changes (42). The mechanisms of 5-HT antinociception may be through direct inhibition of postsynaptic cells that are in the pain pathway such as STT neurons or activation of inhibitory interneurons that synapse onto STT cells. For example, 5-HT can alter responses mediated by NMDA, quisqualate, and kainate (76). Additionally, wide dynamic range projection neurons made hyperexcitable after SCI possess 5-HT receptors (38, 107) which are subject to modulation (98). Both heterocyclic (47) and tricyclic antidepressants (78, 84), agents which inhibit serotonergic reuptake, have been shown to produce analgesia in a variety of situations, effects thought to be mediated by dorsal horn 5-HT_T. Selective serotonin reuptake inhibitors are also capable of producing antinociception as shown by the present study.

In summary, our results demonstrate the plasticity of 5-HT and 5-HT_T systems as well as the importance of 5-HT modulation in attenuation of allodynia and hyperalgesia. We also demonstrate that low thoracic spinal lesions contribute to bilateral behavioral changes in forelimbs and hindlimbs. Furthermore, the 5-HT responses demonstrate denervation supersensitivity in a model of SCI and indicate that local delivery of compounds near the lesion attenuate the bilateral allodynia and hyperalgesia in both forelimbs and hindlimbs. We suggest the existence of altered hyperexcitability through a spinal pain pattern generator (54, 58) near the lesion site and that compounds delivered in the perilesion region may prove useful in treating altered 5-HT systems after SCI (63).

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