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Restorative effects of stimulating medullary raphe after spinal cord injury

Ian D. Hentall, PhD;* Scott S. Burns
The Miami Project to Cure Paralysis, Department of Neurological Surgery, University of Miami Leonard M. Miller School of Medicine, Miami, FL

Abstract—Serotonin in the spinal cord acutely modulates nociceptive transmission and motor reflexes and may also assist functional restoration after spinal cord injury (SCI). It is released there mainly by descending axons of the medulla's nucleus raphe magnus (NRM). We examined whether mechanical allodynia (cutaneous hypersensitivity) after incomplete SCI is sustainably reversed by prolonged, intermittent electrical stimulation of the NRM and whether altered NRM activity accounts for the allodynia. NRM stimulation was given to rats over several days (average 3.2), beginning about 1 hour after moderate thoracic contusion injury. This stimulation reduced mechanical allodynia in forepaws but not hindpaws at 6 weeks after injury (vs nonstimulated controls). Histologically, the stimulation augmented white matter and reduced astrocytosis (glial fibrillary acidic protein immunostaining) in injured segments at 15 weeks. Cavity volume and perilesion neuron numbers were unchanged. Single-cell extracellular recording 12 to 14 weeks after thoracic contusion injury revealed generally higher spontaneous firing and weaker responses to above-injury noxious stimulation in both inhibited and excited NRM neurons; unresponsive neurons were fewer. Neurons inhibited from dermatomes above the injury were excited from below. Altered NRM activity is unlikely to cause SCI allodynia, since inhibited and excited classes are believed to oppositely modulate nociception. Prolonged, early NRM stimulation probably reverses above-injury allodynia by facilitating qualitative recovery of remaining tissue.

Key words: allodynia, deep brain stimulation, GFAP, myelination, pain, raphe magnus, rat model, regeneration, rehabilitation, spinal cord injury, thoracic contusion.

INTRODUCTION

Motor function after an incomplete spinal cord injury (SCI) typically shows some degree of stable recovery in subsequent months. In contrast, on the sensory side, spontaneous pain occurs in about two-thirds of patients with chronic SCI, often first appearing many months later [1–2]. The factors allowing motor recovery, such as sprouting of collaterals or regrowth of intact descending inhibitory fibers below or at the injury level, are presumably insufficient to restore normal pain sensation. Thus pathological processes, possibly including atrophy, apoptosis, gliosis, and aberrant sprouting [3–5], can be assumed to predominate, causing a net result of increased activity in ascending nociceptive pathways. In order to understand and optimize the treatment of pain in SCI patients, therefore, more needs to be known about the status and effect of different nociceptive and nociception-modulating pathways after SCI, in particular about which pathways recover or assist recovery and which remain benign or become pathological.

Abbreviations: 5-HT = serotonin, ANOVA = analysis of variance, BBB = Basso-Beattie-Bresnahan, DBS = deep brain stimulation, GFAP = glial fibrillary acidic protein, IgG = immunoglobulin G, NRM = nucleus raphe magnus, PBS = phosphate-buffered saline, SCI = spinal cord injury, SEM = standard error of the mean, T = thoracic.

***Address all correspondence to Ian D. Hentall, PhD; University of Miami, PO Box 016960, Miami, FL 33101-6960; 305-243-9846; fax: 305-243-3921.**
Email: ihentall@med.miami.edu
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Several descending pathways from the brainstem provide critical control of the transmission of nociceptive signals in neurons of the mammalian spinal cord [6–7]. A major descending projection originates in the nucleus raphe magnus (NRM) and adjacent regions of the rostral ventral-medial medulla. The axonal terminals of NRM neurons release most of the serotonin (5-HT) found in the spinal cord [8] and, conversely, many cell bodies of NRM neurons show immunostaining for 5-HT [9–10]. Some NRM neurons called off-cells are inhibited during acute application of a noxious, thermal, or mechanical stimulus, and this pause in firing is essential for occurrence of withdrawal reflexes, as has been shown in lightly anesthetized rats [11–12]. This observation and other findings, including the excitation of off-cells by systematically applied μ-opioids when withdrawal reflexes are suppressed, have led to the proposal that off-cells mediate analgesia [13–15]. Other NRM neurons called on-cells are excited by acute noxious stimulation. On-cells are inhibited by μ-opioids, which, along with other correlations observed between firing rate and various hyperalgesic phenomena, suggests that they are pronociceptive [15–17]. A third cell class, neutral cells, is unaffected by noxious stimulation or morphine [16,18]. Descending serotonergic fibers from the NRM also play a major role in setting up movements [19–20] and controlling sympathetic outflow [21]. For example, activating cell bodies in the NRM of decerebrate cats can produce treadmill locomotion [22]. Indeed, it seems best to regard the motor function of the NRM as primary so that the suppression of nociception and activation of sympathetic autonomic output may be viewed as serving an accessory role in preparing the organism for locomotion [20,23–24].

In addition to its immediate effect as a neurotransmitter, 5-HT has other neurobiological functions, especially neurotrophic and neuroprotective effects [25]. These influences suggest that the NRM-spinal system should have a restorative effect after injury. Since this hypothesis has not been directly tested before, we report here some effects of electrically stimulating for several days the NRM of rats with or without incomplete thoracic SCI. A specially built wireless cranial implant delivered a complex daily pattern of intermittent stimulation. The consequences many weeks after SCI for allodynia and for certain histological variables are presented. A major concern in this study was that firing in the NRM might be altered after SCI, which would have an important bearing on how the effects of chronic stimulation might be interpreted. Indeed, since NRM neurons modulate nociception, alterations in their firing could potentially contribute to the allodynia of SCI. We therefore also report some separate, previously unpublished work on recordings of single-neuron responses in the NRM of rats with chronic incomplete thoracic SCI.

METHODS

Design Overview

Three main types of experiments were performed. All experiments were approved by the Institutional Animal Care and Use Committee of the University of Miami Leonard M. Miller School of Medicine (protocols 06-009 and 06-132).

1. The effects of chronic NRM stimulation on nociception and anatomical recovery from SCI were investigated in adult female Sprague-Dawley rats (Charles River Laboratories; Wilmington, Massachusetts) with moderate thoracic contusion injuries. The same injury method was used on adult female rats in a prior successful study of a combination treatment for SCI [26], thus allowing direct comparison. In addition to the stimulated treatment group, two kinds of injured controls were used: rats whose implanted stimulators and microelectrodes were never turned on and rats without implants. A number of motor tests were performed weekly but will not be described further in this article, whose focus is nociception. Allodynia of the limbs was measured 40 to 47 days after injury. Euthanasia at 15 weeks was followed by histological processing of brain and spinal cord. Quantitative analysis was performed on several anatomical variables.
2. The influence of chronic NRM stimulation on nociception in the absence of injury was studied in four adult female Sprague-Dawley rats (source as above). These trials constituted the pilot safety studies of the newly designed stimulator, which were required before its use in injured animals. A wireless, self-powered stimulator was mounted dorsally on the skull, from which an integral stimulating microelectrode protruded into the brainstem target, as specified subsequently. Controls had implanted stimulators and microelectrodes that were never turned on. The tail-flick test of thermal nociception was performed on different days in the first several weeks after implantation.
3. Single-neuron activity was recorded in the NRM of adult male Fisher rats (Harlan Laboratories; Tampa, Florida) anesthetized with intravenously infused pentobarbital.

Male rats of Fisher and Sprague-Dawley strains have given rise to most of the basic data on neurophysiological responses in the NRM [27–28]. The animals either had moderate thoracic contusion injuries made 12 to 14 weeks previously or served as sham-operated, noninjured controls. Open-field motor performance was assessed with the Basso-Beattie-Bresnahan (BBB) test 1 and 12 weeks after injury [29]. Responses of neurons to noxious mechanical stimuli were examined above and below the segmental level of the injury in nonsurvival experiments.

The location of the microelectrode used for recording or stimulating in the brainstem was always verified histologically in coronal sections stained with cresyl violet. Animals were randomly assigned to all treatment groups. Statistical tests were performed with commercial software (SPSS version 15.0, SPSS Inc; Chicago, Illinois).

Stimulator and Electrodes

The silicone-encapsulated stimulator, built in-house, included a microprocessor and a step-up direct current converter (40 V compliance) powered by two 1.5 V silver oxide batteries in series. It was 20 mm long, 9 mm wide, and 5 mm high and weighed approximately 2 g. An infrared emitting diode communicated its state to a custom detector, and a magnet held nearby could turn off the stimulation and control time parameters. A parylene-insulated tungsten microelectrode (catalog number 573210, A-M Systems Inc; Carlsborg, Washington) was integrated with the encapsulated stimulator, protruding from it directly. This microelectrode had a factory-specified resistance of 0.5 MΩ at 1 KHz and a noninsulated surface of about 3,400 μm^2. The anode was made of stainless steel wire (50 µm diameter). The microprocessor was programmed to give 5 minutes of 8 Hz stimulation alternated with 5 minutes of rest for 12 daytime hours, followed by 12 nocturnal hours of rest. The output consisted of fixed-amplitude, 30 µA cathodal pulses, with a default width of 1.0 ms. The estimated charge injection for each 1 ms pulse was 0.9 mC/cm^2. A 30 µA stimulus can be estimated to activate NRM neurons near their cell bodies for a radius of 0.18 mm, given a threshold-distance relation of around 850 $\mu A/mm^2$ [30]. Previous electrochemical measurements of spinal 5-HT release by NRM stimulation indicate that this stimulus liberates a high level of 5-HT and that the 5-minute rest allows the activated neurons time for recovery [31]. The purpose of turning off the stimulation for 12 hours each night was to mitigate disruption of diurnal arousal cycles.

Implantation of Stimulator and Electrodes

Implantation in the chronic injury study was done either within 60 minutes of the injury or 5 to 7 days before the injury to permit evaluation of behavioral effects of the implant itself. In both cases, prolonged stimulation was commenced 30 minutes to 1 hour after the injury. Rats were anesthetized with a combination of intramuscular ketamine (40 mg/kg) and xylazine (5 mg/kg) and placed in a stereotaxic holder. Holes were drilled in the skull above the midline stereotaxic target and 2 mm bilaterally; miniature stainless steel screws were inserted into the latter to grip the dental acrylic that secured the implant. The anode lead was wrapped around one of the skull screws. The microelectrode was lowered stereotaxically to the NRM target: 2.4 to 2.0 mm caudal and ±0.5 mm of the interaural line during activate stimulation. Passage through the dorsal medulla was accompanied by synchronized bilateral facial twitches, which were used to verify accurate placement. The final target was always just below (<1 mm) where the twitches were no longer elicited. Two animals showed stimulus-evoked movement on awakening from the anesthesia, possibly due to pyramidal tract activation. Their stimulators were promptly inactivated, and they were reassigned to the implant-control group. Postoperative care after implantation was the same as for the spinally injured rats described subsequently.

Spinal Cord Injury

The contusion injury for the study of single-neuron responses in the NRM was created with the Electromagnetic SCI Device developed at Ohio State University [32]. The anesthetic was ketamine plus xylazine, as just described for the previous experiment. Following anesthesia and retraction of superficial muscle and skin, a laminectomy was performed at the ninth thoracic (T9) vertebra. Clamps on adjacent vertebrae stabilized the spinal column. A 4 mm circular impactor tip displaced the dorsal surface of the spinal cord a distance of 0.95 mm at a force of approximately 3 kdyn for 20 ms.

For the chronic stimulation study, initial injuries were made with a New York University MASCIS (Multicenter Animal Spinal Cord Injury Study) impactor at segment T8. The rats were anesthetized with ketamine plus xylazine (doses described earlier) if the implant and injury were done in the same session or anesthetized with isoflurane (1.2%) if implantation was done 5 to 7 days earlier. The injury was made by dropping a 10 g rod from a height of 12.5 mm. Impact depth, velocity, and compression force were monitored to ensure they remained

within range. This injury was almost equivalent to the injury made for the recording study reported in the present article, since both methods give a similar time course of motor recovery [29].

After surgery, the rats were allowed to recover on a warmed blanket with ready access to water and food. Gentamycin (5 mg/kg intramuscular) was given for 3 days after surgery to control infection. Buprenorphine (0.01 mg/kg subcutaneous of 0.3 mg/mL Buprenex) was given on the postsurgery day and on 2 subsequent days to control pain. The bladder was expressed daily by hand until the animal had recovered control of voiding.

Behavioral Tests

Mechanical allodynia on the plantar surface of all paws was tested by means of calibrated manual von Frey filaments. The rats were positioned on a wire grid and approached from below. The threshold for paw withdrawal was determined by the 50 percent threshold method [33] in three consecutive trials for each paw and then averaged bilaterally. Tail-flick latencies were measured with a commercial device (Basile Tail-Flick Unit, Stoelting Co; Wood Dale, Illinois). Latencies were measured in seven or eight trials, at 5 minutes apart, each trial consisting of three readings separated by 30 seconds. Open-field motor behavior was quantified according to the BBB method [29] by trained observers who were unaware of the state of activation of the stimulator. The BBB score could range between 0 (worst performance, complete spinal shock) and 21 (normal performance).

Single-Cell Recording

Recordings were made under pentobarbital anesthesia (constant intravenous infusion, 0.30–0.42 $mg \cdot kg^{-1}h^{-1}$) through commercial stainless steel microelectrodes with electroplated platinum-black tips (2–4 MΩ impedance at 1 kHz, 125 µm shank diameter; FHC Inc; Bowdoin, Maine). The microelectrode was moved slowly to the point of maximum amplitude for a recorded cell. Recording sites were never more than 100 µm apart to avoid repeat sampling. A single recording site yielded 1 to 4 units, which were sorted offline by a routine written in MATLAB (The MathWorks; Natick, Massachusetts). The spikes were discriminated based on k-means clustering of their first and second principle components. Stimulation was applied by toothed forceps attached to an electrical position transducer. Test points on the body included the left and right fore- and hindpaws; the dorsal tail (middle); and the middle of the back above, below, and at the dermatome corresponding to the spinal injury level. Neurons were classified as inhibited, excited, or unaffected by noxious stimulation applied at dermatomes above the lesion. These classes were respectively designated as off-cells, on-cells, and neutral cells [11,27,34]. At the end of several mapping tracks, the microelectrode's position was marked with an electrolytic lesion by anodal 5 V pulses of 20 ms duration given for 10 seconds at 25 Hz. Then, the animal was perfused as described below.

Anatomical Analysis

After euthanasia with pentobarbital overdose (100 mg/kg intraperitoneal), phosphate-buffered saline (PBS) (pH 7.6, 4 °C) was perfused intracardially and followed by 4 percent paraformaldehyde in 0.1 M PBS (pH 7.6, 4 °C). The extracted spinal cord was photographed under identical conditions of illumination and orientation to analyze treatment-related differences in gross appearance with Metamorph software (Molecular Devices; Downingtown, Pennsylvania).

Paraffin-embedded tissue ±5 mm around the lesion was cut sagittally at a 12 µm width. Standard immunostaining procedures were used to localize glial fibrillary acidic protein (GFAP) in every tenth section. Sections were incubated in anti-GFAP antibody (1:500; Chemicon catalog number AB5804, Millipore; Billerica, Massachusetts) overnight at 4 °C. The sections were then washed in PBS and incubated for 2 hours at room temperature in fluorophore-labeled antirabbit immunoglobulin G (IgG) antibodies (1:200; Molecular Probes catalog number A21206, Invitrogen; Eugene, Oregon). Additionally, paraffin-embedded tissue between 5 and 10 mm rostral and caudal to the lesion was cut coronally at 12 µm and every twentieth section was similarly immunostained in antineuronal nuclei NeuN antibody (1:1000; Chemicon catalog number MAB377, Millipore) and fluorophore-labeled antimouse IgG antibodies (1:200; Molecular Probes catalog number A11005, Invitrogen). Neurolucida software (MBF Bioscience; Williston, Vermont) was used to create unbiased counts of neurons in the dorsal (I–IV), intermediate (V, VI, X), and ventral (VII–IX) laminae in five consecutive coronal sections. Cavity and contusion volumes were reconstructed by Neurolucida software from analysis of all sagittal sections. The volumes of gray and white matter with abnormal appearance and the volume of the empty cavity were measured by manually outlining their boundaries for each section [35]. An assessment of staining intensity was obtained from the rating (on a 1–10 scale) of a naïve observer.

EXPERIMENTAL RESULTS

Effects of Stimulator Implants and Analgesia in Normal Rats

The stimulator-microelectrode combination was implanted into 27 rats that subsequently received a moderate thoracic contusion injury and in 4 pilot rats without SCI. In 13 rats, the implant was not activated beyond the period of awakening from surgical anesthesia. A further 6 rats were injured identically but received no implant, and 6 rats were used to measure allodynia in normal controls (noninjured, nonimplanted). The stimulators regularly lasted 3 weeks or longer before battery exhaustion ex vivo but often failed in vivo after a few days of operation (mean lifetime 3.2 days). Among likely explanations for the higher rate of failure in vivo are leakage of fluid into the electronics and program halt because of transient power drop during a stimulus pulse.

Overt acute effects of the stimulator implant were never seen. Indeed, without use of the handheld infrared detector, it was impossible to tell whether the stimulator was off or was applying pulses. However, the BBB score in noninjured animals that had received implants on the previous day was 18.5 ± 0.53 standard error of the mean (SEM) (n = 15) compared with the normal values of 21 measured in untreated rats, which was significantly different (t-test, $p < 0.01$). (All data are shown as mean ± SEM unless otherwise indicated.) This disruptive effect of the implant per se apparently disappears by a few weeks, since the BBB score at 3 weeks differed little between implant-control rats (11.13 ± 0.29) and nonimplanted rats (10.75 ± 0.13).

Four rats with implants, two of which served as nonstimulated controls, were subjected to the tail-flick test. Daily monitoring with the remote detector indicated that the stimulation lasted 5 to 7 days. The testing, in which a heat stimulus was applied to a small spot on the tail, was performed on 4 days between the 7th and 15th day after implantation, when stimulation had already permanently ceased. On all 4 days, including when higher heat intensities were used on the 2 later days, reaction latencies were prolonged in the two animals whose stimulators had been active (**Figure 1**).

Effects of Chronic Nucleus Raphe Magnus Stimulation on Nociception

Compared with the noninjured, untreated rats, allodynia was evident in both the forepaws and the hindpaws of rats roughly 6 weeks after moderate T8 contusion injury (with or without implanted stimulators). This finding is similar to what has been noted previously in, for example, rats with T13 hemisections [36]. Giving several days of NRM stimulation early after injury reversed the forepaws' allodynia at 6 weeks so that the average von Frey threshold approached normal (**Figure 2**). Significance was assessed by Dunnett t-test (two-sided) post hoc comparison of bivariate analysis of variance (ANOVA), with the reference group being the implant-control group. The comparison with the stimulated group for forepaw allodynia but not hindpaw allodynia showed a significant difference (forepaw: $p = 0.007$, hindpaw: $p = 0.28$). Comparisons with the nonimplanted, noninjured group showed a significant difference from the injured implant-control group for both fore- and hindpaws (hindpaw: $p = 0.002$, forepaw: $p = 0.04$). Linear regression of mean bilateral forelimb withdrawal/vocalization threshold versus number of days of stimulation was also significant ($p = 0.025$, $F_{1,19} = 5.93$).

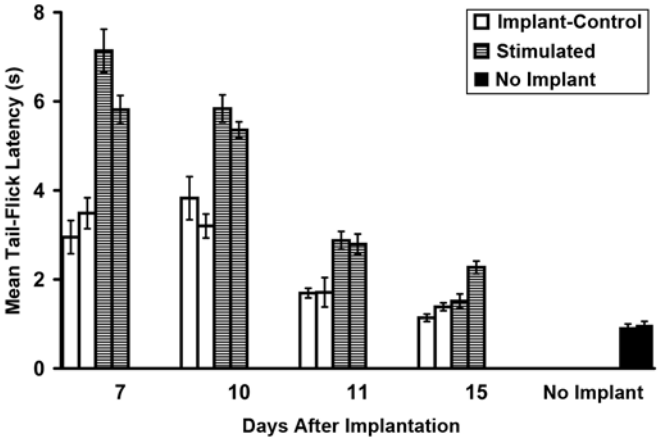

Figure 1.
Analgesia due to prolonged nucleus raphe magnus (NRM) stimulation in noninjured rats. Withdrawal latency (tail-flick) was measured in response to barely noxious, escapable heat stimulus on tail. Data from four rats are shown individually. Two rats ("stimulated") received stimulation of NRM for several days that ceased before first tail-flick test on day 7 after implantation of electrode and stimulator. Two others ("implant-control") received implants but not prolonged NRM stimulation. The third pair ("no implant") consisted of normal rats matched for sex, age, and breed. Higher heating lamp intensity on days 11 and 15 confirmed that difference was not dependent on stimulus intensity. Latencies were measured over seven or eight trials. Each trial consisted of three consecutive readings taken 30 s apart. Error bars indicate standard error of the mean within each animal.

Effects of Chronic Nucleus Raphe Magnus Stimulation on Anatomical Recovery

The injury zone of the thoracic spinal cord extracted after euthanasia at 15 weeks had a more myelinated appearance, that is, whiter and less translucent, in the rats that had received prolonged NRM stimulation than in the implant-control group (**Figure 3**). This finding was confirmed by luminosity measurements made on dorsal views extending ±0.2 mm around the central point of the lesion: 24.0 ± 1.2 versus 18.9 ± 0.7 intensity units/mm^2 (unpaired two-tailed *t*-test, $p < 0.005$).

Cavity size showed considerable variation between groups but with no significant difference between stimulated rats (0.29 ± 0.11 mm^3, $n = 12$) and implant-control rats (0.21 ± 0.11 mm^3, $n = 8$). The volume of contused tissue, defined by visual evidence of cellular or axonal degeneration within the tissue, was likewise not significantly different (stimulated rats: 4.8 ± 1.2 mm^3, implant-control rats: 5.0 ± 1.0 mm^3). The total volume in 1 cm lengths of sagittally sectioned tissue also differed insignificantly (stimulated rats: 10.8 ± 1.2 mm^3, implant-control rats: 9.2 ± 1.4 mm^3).

Chronic stimulation had no effect on the number of cells marked by NeuN immunostaining in dorsal, intermediate, or ventral laminae, either rostral or caudal to the lesion, compared with the control implant (**Table**). However, GFAP, examined in two sagittal sections taken 0.5 mm bilateral from the midline in each rat, showed noticeably less immunostaining around the lesion site in the stimulated rats than in the rats belonging to the implant-control group (**Figure 4**). The average rating made by the naïve observer on the 1–10 scale was 3.44 ± 0.65 ($n = 8$) for the implant-control rats and 2.42 ± 0.34 ($n = 12$) for the stimulated rats.

Neuronal Activity in Nucleus Raphe Magnus of Rats with Spinal Cord Injury

Recordings were obtained from three sham-injured rats and three rats with 12-week bilateral BBB scores of 12 to 13. One additional rat ("mildly injured") had full bilateral recovery at 12 weeks, with BBB scores of 14 at 1 week and 21 at 12 weeks, probably due to an unintentionally weak impact at injury. A total of 31 on-cells, 33 off-cells, and 23 neutral cells were analyzed. In rats with moderate SCI, 20 on-cells, 23 off-cells, and 8 neutral cells were sampled. In sham-injured rats, 11 on-cells, 10 off-cells, and 15 neutral cells were sampled. The reduced

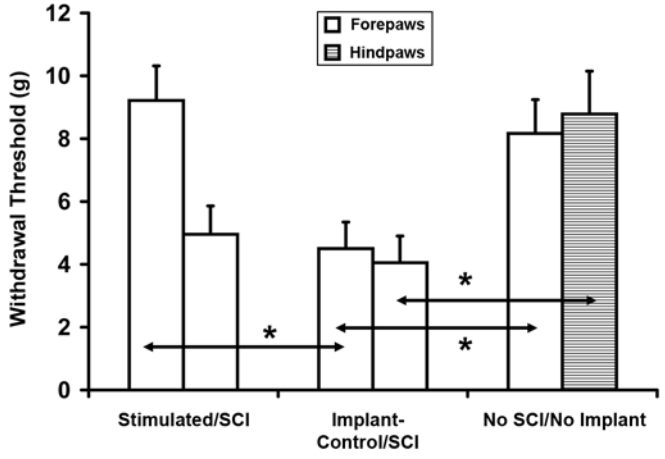

Figure 2.
Paw allodynia, measured with von Frey filament, 40–47 days after moderate thoracic spinal cord injury (SCI). Several days of nucleus raphe magnus stimulation early after injury ("stimulated/SCI") reversed injury-induced allodynia in forepaws, with average von Frey threshold returning to nearly normal. *All post-hoc comparisons against implant-control/SCI group were significant at $p < 0.05$ by two-sided Dunnett *t*-test, except for hindpaw allodynia in stimulated rats. Error bars indicate standard error of the mean.

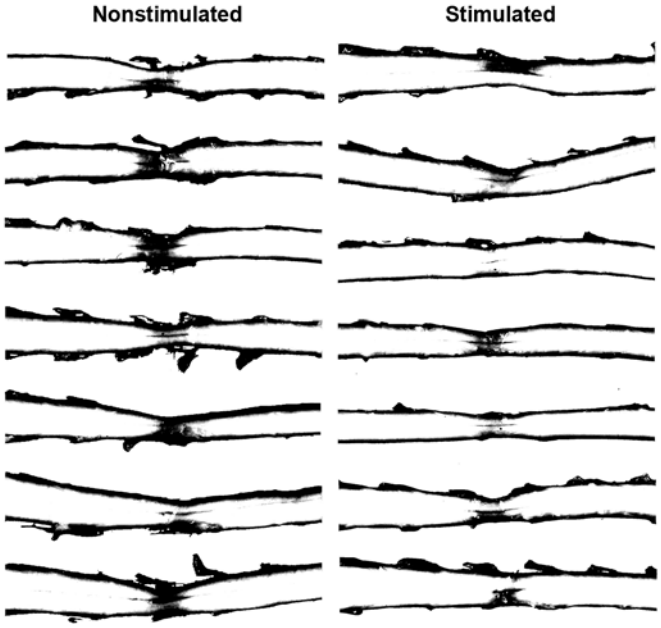

Figure 3.
Photomicrographs showing dorsal view of injury zone in thoracic spinal cord at 15 weeks postmortem. All images were taken with identical camera settings and lighting. Image contrast has been sharpened to better reveal dark zone of injury, where tissue lacks myelin. This zone extends completely across injured segment in most implant-control rats but only in one rat that received nucleus raphe magnus stimulation.

Table.
Gross cell numbers and morphological status in and near lesioned spinal segments of stimulated and implant-control (nonstimulated) rats. Neuron counts were measured in coronal sections approximately 0.75 cm rostral and caudal to center of lesion. Volumes of injury-induced cavities and of remaining, damaged-appearing tissue were obtained from 1 cm sagittal sections centered on lesion. Data shown as mean ± standard error of the mean.

Parameter	Stimulated	Nonstimulated
Cell Density (per mm^3)		
Laminae I–IV		
Rostral	117.0 ± 9.3	135.0 ± 10.0
Caudal	128.0 ± 13.0	132.0 ± 15.0
Laminae V–VI, X		
Rostral	52.0 ± 4.1	53.0 ± 4.9
Caudal	60.0 ± 3.7	57.0 ± 7.3
Laminae VII–IX		
Rostral	11.1 ± 1.3	10.8 ± 1.3
Caudal	11.6 ± 1.3	10.4 ± 1.5
Injury Cavity (mm^3)	0.29 ± 0.11	0.21 ± 0.11
Damaged Tissue (mm^3)	4.8 ± 1.2	5.0 ± 1.0

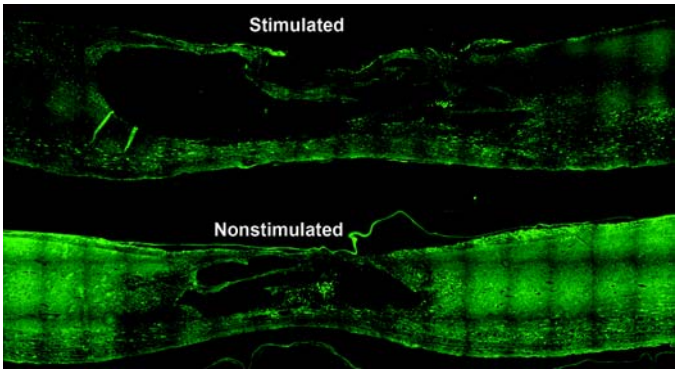

Figure 4.
Reconstructed high-magnification photomicrographs of sagittal sections 0.5 mm from midline, immunostained for glial fibrillary acidic protein (GFAP). Sections are from representative stimulated and implant-control (nonstimulated) rats, showing typical injury cavities and differences in GFAP staining.

frequency of neutral cells following SCI was statistically significant (chi-square, $p = 0.02$).

Spontaneous activity was higher in off-cells of moderately injured rats (10.4 ± 2.3 spikes/s) than in the sham group (3.6 ± 0.5 spikes/s). The mildly injured rat's off-cells fell between these values, with spontaneous activity of 7.9 ± 0.8 spikes/s. On-cells showed little spontaneous activity in the mildly injured rat or the sham group, being respectively 0.43 ± 0.12 spikes/s and 0.67 ± 0.32 spikes/s. On-cells did show higher spontaneous activity in the moderately injured rats (9.0 ± 2.0 spikes/s). Neutral cells had similar rates of spontaneous activity in injured and sham groups (10.6 and 9.1 spikes/s, respectively.)

Sites of application of the noxious mechanical stimulus were divided into dermatomes above-, below-, and at-lesion. Spikes were counted in 2-second periods just before stimulation and during stimulation (**Figure 5**). The moderately injured rats manifested a complex set of responses in presumed off-cells. For example, a finding of excitation of the back below the injury level, inhibition above, and strong excitation at the injured dermatome was common (**Figure 6**). Major differences between off-cell responses above and below the injury level were not seen in the control group or in the rat with the mild injury. The moderately injured rats displayed weaker average off-cell responses in upper dermatomes than did sham-injured rats (35% ± 3%, $n = 80$ trials, vs 59% ± 11%, $n = 49$ trials). This group's on-cell responses were also weaker on average, with the increase in firing being 0.90 ± 0.24 spikes/s versus 2.10 ± 0.40. In sum, quantitative changes in neuronal activity emerged in the NRM of rats with chronic SCI, along with some divergence from the normal proportions of the nociceptive response classes.

DISCUSSION

General Significance

Our main finding is that electrical stimulation of the NRM for a few days just after SCI reversed postinjury allodynia in the forelimbs, although hindlimb allodynia remained unalleviated. The prolonged NRM stimulation also produced thermal hypoalgesia lasting at least 2 weeks in noninjured animals. Long-term improvement in above-level somatosensation after SCI was accompanied by improvements in several aspects of motor performance, which are described in a recent abstract [37] whose details will appear elsewhere. The NRM stimulation caused a concomitant increase in the quality of surviving tissue around the injury zone, specifically a visible increase in white matter and a decrease in astrocytosis. On the other hand, gross anatomical variables, such as the volume of the injury cavity and the number of neurons in the vicinity of the injured segments, did not improve.

The enhancement of recovery by prolonged NRM stimulation raises the question of whether deep brain stimulation (DBS) can be exploited to treat some patients with incomplete SCI. The human NRM is apparently

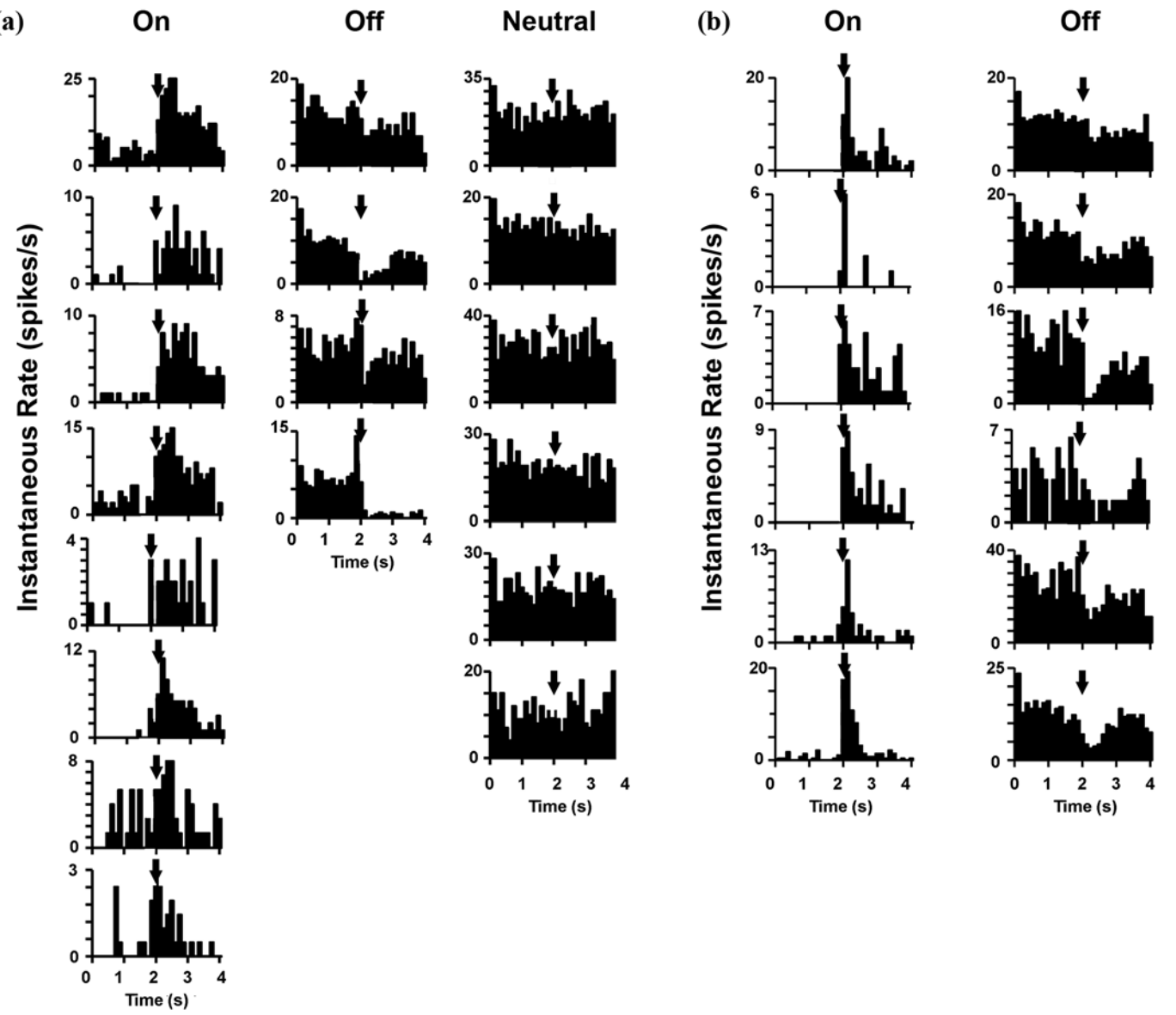

Figure 5.
Histograms of single-neuron action potential activity in on, off, and neutral cells found by systematic microelectrode sampling in nucleus raphe magnus and recorded during noxious mechanical stimulation of above-injury dermatomes. Stretches of 4 s of recorded activity are displayed in 0.125 ms bins. Noxious mechanical pressure was applied for several seconds, starting at 2 s as indicated by arrows. **(a)** Sham-operated control. **(b)** Rat with mild spinal cord injury, in which no neutral cells were discovered.

ruled out as a practical target because it is very difficult to approach neurosurgically and presents too great a risk, being near vital centers for breathing and cardiovascular control. However, it receives a strong excitatory projection from the periaqueductal gray [38], which has been exploited in about a thousand patients so far to alleviate otherwise refractory pain by DBS [39]. Our preliminary work in rats has shown motor recovery from prolonged stimulation of the periaqueductal gray region [37].

Consequences of Spinal Cord Injury for Activity and Function of Nucleus Raphe Magnus Neurons

Plasticity of neuronal responsiveness following midthoracic hemisection or contusion has previously been

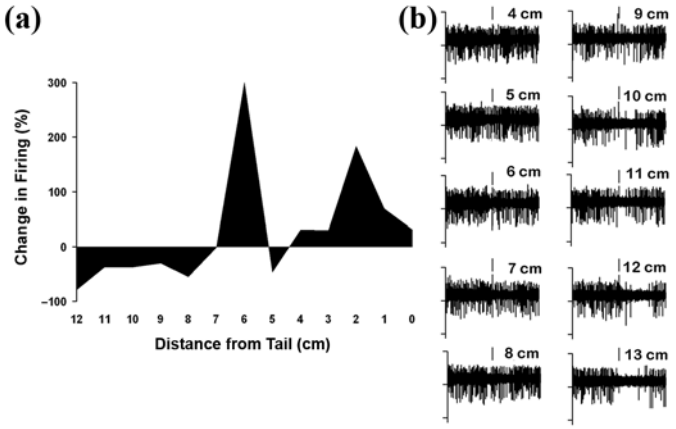

Figure 6.
Dependence on dermatomal segment of off-cell responses in moderately injured rats. **(a)–(b)** Examples from two neurons. Skin of back was squeezed by forceps near midline at randomly ordered points spaced 1 cm apart along its length. Distance from tail is shown in centimeters. Dermatome corresponding to center of injury was at 6 cm. **(a)** Average responses from one neuron, in which percent change was calculated from firing rate in 2-second periods just before (B) and after (A) skin stimulus, according to formula 100(A–B)/B. **(b)** Raw recordings created as described in **Figure 5**.

observed in areas of the medullary reticular formation lateral to the NRM [40–41]. The midline NRM itself after SCI has not been reported on before. However, plasticity has been seen in the NRM, as well as in contiguous areas during cutaneous inflammation [42–45] and peripheral neuropathy [46–47]. The neuronal recordings described in this article demonstrated that after moderate thoracic SCI, the three major NRM cell types classified by responses to acute noxious stimulation [11,27,34] continue to be present. However, the responsiveness of both on-cells and off-cells to noxious mechanical stimulation was diminished, their spontaneous activity was elevated, and a relative paucity of neutral cells emerged. Rats with chronic inflammation of the hindpaws produced by injection of complete Freund's adjuvant have been reported to show interesting parallels to these findings: first, noxious thermal stimulation of the hindpaws elicited weaker-than-normal off-cell responses in the NRM and, second, most neutral cells became "off-like" or "on-like" several hours after injection of the adjuvant [45] and remained so for at least 24 hours. In the present study, neurons inhibited by noxious stimulation in dermatomes above the injury were seen to be excited below, which, from the standard model for off-cells [27–28], implies an augmented antinociceptive effect if their axons penetrate below the injury. However, since on-cells and off-cells are thought to have opposite effects on nociception, whereas their activity is changed in the same direction by SCI, a major net contribution to allodynia appears unlikely.

Effects of Prolonged Nucleus Raphe Magnus Stimulation on Injured Spinal Cord

After midthoracic SCI, we found that the rats had mechanical allodynia in both their hindlimbs and forelimbs, as has been observed previously after either hemisection or contusion injury at this level [36,48]. The below-level effect may quite possibly be due to loss of descending inhibition from the NRM and elsewhere [7,36,49–50], which might explain why it was not reversed by chronic NRM stimulation. The above-injury allodynia is harder to explain, although it may be relevant that transplantation of embryonic raphe cells that release 5-HT into the thoracic injury zone has been shown to reverse allodynia at both forelimb and hindlimb levels [46].

The hypothesis underlying the present work was that prolonged NRM stimulation would have multiple beneficial actions, mainly due to several effects of 5-HT released in the spinal gray matter but possibly supplemented by activity-dependent sprouting of raphe-spinal terminals or unmasking of silent synapses. The short-term effects of 5-HT in enhancing motor output [19] and reducing nociception [7], if maintained by prolonged electrical stimulation of the NRM, could enhance the normal course of rehabilitation. Serotonin-containing axons in the spinal cord are relatively resistant to injury [51], while 5-HT itself has been shown to have neuroprotective and neurotrophic effects in many systems [25,52], including the mammalian spinal cord. 5-HT is reported to be contained in about 50 percent of descending NRM neurons in the rat [9]. The neurotransmitter is present in most, if not all, spinal segments and laminae, which reflects the extensive branching of raphe-spinal axons [53–55]. All three main types of NRM cells contribute about equally to the descending serotonergic projection [10]. In sensory laminae, 5-HT acutely inhibits ascending nociceptive transmission [56]. Nociceptive processing after SCI is apparently more complicated; inhibition of below-level spinal nociceptive neurons by 5-HT has been found to be reversed by both 5-HT1A and 5-HT3 antagonists [57], but spinal 5-HT3 receptors have also been shown to facilitate at-level allodynia after SCI [58–59]. In locomotor control, 5-HT7 and 5-HT2A receptors have major roles [60], generally enhancing locomotor pattern generation in the spinal cord. For example, treadmill locomotion is improved acutely by intrathecal administration

of the 5-HT2 agonist quipazine in several mammalian species with chronic spinal transection [61–62]. After a thoracic injury, the amount of 5-HT in the dorsal horn increases at and above the injury level [63], which could be the basis for 5-HT3-receptor mediated allodynia [58–59,64]. Below the level of injury, the 5-HT concentration falls, but some compensatory sprouting of spared axons appears to occur [65].

Successful model therapies for SCI are often associated with pronounced regrowth of serotonergic fibers. For example, anti-CD11d integrin antibody has been shown to promote regrowth distal to a thoracic contusion injury, in parallel with reduced nociception and improved locomotion [66]. Also, when the Nogo-66 receptor, which inhibits axonal growth, is blocked with intrathecal NgR(310)ecto-Fc protein after midthoracic bilateral dorsal hemisection, improved locomotion is accompanied by increased sprouting of both raphe-spinal and corticospinal axons [67]. Cell or tissue grafts, such as Schwann cells, olfactory ensheathing glia, or olfactory lamina propria, placed in the vicinity of a spinal lesion can also induce increases in 5-HT-containing terminals [68–69]. This regrowth may be linked to a relative resistance of NRM axons to injury. Retrograde transport of label introduced distal to an injury reaches a high proportion of NRM cell bodies [51,70]. Increased serotonergic raphe-spinal sprouting has been seen after low thoracic hemisections in double-knockout mice lacking GFAP and vimentin (another astrocyte-specific protein) [71], along with fewer reactive astrocytes. Hence, the reduced reactive astrocytosis in an injured segment of NRM-stimulated rats, as shown by reduced GFAP staining, could be directly related to the mechanisms of enhanced functional and anatomical restoration produced in the present experiments by NRM stimulation.

Immortalized raphe cells secreting both 5-HT and brain-derived neurotrophic factor reduce nociception and improve locomotion when implanted in the subarachnoid space after thoracic hemisection [36]. The success of engrafted embryonic raphe cells in improving locomotor recovery in rats with SCI has been proposed to be due to 5-HT2-mediated connections [72]. Stimulation of either 5-HT1A or 5-HT2 subtype receptors promotes plasticity and motor improvement in the transected spinal cord [50,73]. Astrocytes' 5-HT1A or 5-HT2 receptors apparently mediate some of the diverse neurotrophic effects of 5-HT, including cell proliferation or maturation, synaptogenesis, and apoptosis [25]. Astrocytes are also controlled by ATP (adenosine 5'-triphosphate) released from firing neurons, leading them in turn to release cytokine leukemia inhibitory factor, an important trophic factor [74]. Hence, both increased firing per se and excess release of 5-HT by prolonged stimulation of the NRM could alter astrocytes in ways that promote recovery.

CONCLUSIONS

Electrical stimulation of the serotonergic NRM for a few days immediately after SCI improves anatomical and sensory recovery. The effect could be due in part to long-term sequelae of acute motor enhancement and antinociception caused by the additional 5-HT release and could be sustained by the same mechanisms responsible for rehabilitation [75]. The improvement could also be due to the various known neurotrophic effects of 5-HT in the partially injured spinal cord. In addition, since spinally projecting serotonergic fibers have previously demonstrated a superior propensity to regenerate compared with other fiber types, their phenotype may be favorable to activity-dependent induction of new synaptic connections, sprouting of terminals, or unmasking of silent synapses [76–77].

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