Some Autonomic Deficits of Acute or Chronic Cervical Spinal Contusion Reversed by Interim Brainstem Stimulation

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Abstract

Prolonged electrical stimulation of the hindbrain's nucleus raphe magnus (NRM) or of its major midbrain input region, the periaqueductal gray (PAG), was previously found in rats to promote recovery from sensory-motor and histological deficits of acute thoracic spinal cord injury (SCI). Here, some visceral deficits of acute and chronic midline cervical (C5) contusion are similarly examined. Cranially implanted wireless stimulators delivered intermittent 8 Hz, 30–70 μ A cathodal pulse trains to a brainstem microelectrode. Injured controls were given inactive stimulators; rats without injuries or implants were also compared. Rectal distension or squeezing of the forepaws caused an exaggerated rise in mean arterial pressure in injured, untreated rats under anesthesia on post-injury week 6, probably reflecting autonomic dysreflexia (AD). These pressor responses became normal when 7 days of unilateral PAG stimulation was started on the injury day. Older untreated injuries (weeks 18–19) showed normal pressor responses, but unexpectedly had significant resting and nociceptive bradycardia, which was reversed by 3 weeks of PAG stimulation started on weeks 7 or 12. Subsequent chronic studies examined gastric emptying (GE), as indicated by intestinal transit of gavaged dye, and serum chemistry. GE and fasting serum insulin were reduced on injury weeks 14–15, and were both normalized by $\sim$ 5 weeks of PAG stimulation begun in weeks 7–8. Increases in calcitonin gene-related peptide, a prominent visceral afferent neurotransmitter, measured near untreated injuries (first thoracic segment) in superficial dorsal laminae were reversed by acutely or chronically initiated PAG stimulation. The NRM, given 2–3 weeks of stimulation beginning 2 days after SCI, prevented abnormalities in both pressor responses and GE on post-injury week 9, consistent with its relaying of repair commands from the PAG. The descending PAG–NRM axis thus exhibits broadly restorative influences on visceral as well as sensory-motor deficits, improving chronic as well as acute signs of injury.

Keywords: AD; calcitonin gene-related peptide; electrical stimulation; GE; insulin; NRM; PAG

Introduction

SPINAL CORD INJURY (SCI) causes a wide spectrum of sensory, motor, and autonomic deficits that depend on diverse factors, including the severity of the injury and its segmental level.^{1–3} Practical treatments thus need to be comprehensive, addressing many types of injuries and their various signs and symptoms, which calls in turn for broad models of neural damage and repair. The present approach starts from the general premise that local repair processes in traumatized brain or spinal regions are to some degree under the control of a few small brainstem nuclei (raphe nuclei). This idea has received support from several studies that we have conducted over the last decade. For example, when the hindbrain's nucleus raphe magnus (NRM) was stimulated for several days after a recent incomplete thoracic (T8) contusion, a lasting enhancement of sensory and motor recovery ensued, and losses of myelinated fibers in white matter and serotonin-containing fibers in ventral gray matter were reversed.^{4,5} Similarly, when ascending serotonergic systems originating in the midbrain's

dorsal and median raphe nuclei were stimulated in the 1st week after closed-head traumatic brain injury, motor and cognitive performance were enduringly improved, and the anatomical integrity of the neocortex and hippocampus were restored.⁶ The cellular basis of the improved behavior is probably multifactorial.⁷ Beyond mechanical trauma, we recently found that prolonged NRM stimulation in mice attenuates some signs of experimental autoimmune encephalomyelitis, a model of human multiple sclerosis, while blocking production of inflammatory cytokines as well as the underlying myelin-related pathology.⁸

Here, we study some visceral deficits of midline cervical contusion injuries (C5 segment) at both acute and chronic stages, mainly using the midbrain's periaqueductal gray (PAG) as the stimulation target. As we reported previously, PAG stimulation produces a similar set of beneficial effects to NRM stimulation in the rat thoracic (T8) contusion model.⁴ The PAG provides a major non-serotonergic excitatory input to the NRM,^{9,10} and the PAG–NRM–spinal cord axis is commonly viewed as a pain-modulating system.^{11,12} Chronic pain, in theory, provides a useful control

signal endogenous repair systems, which is one of the main concepts underpinning this model of centralized repair control. The PAG has proven to be a safe target in many hundreds of patients receiving deep brain stimulation (DBS) for various kinds of drug-refractory pain,^{13,14} and hence is the more promising target for neurosurgical intervention in human SCI.

Chronic injuries, visceral deficits, and cervical lesions are relatively neglected topics in pre-clinical therapeutic studies; however, addressing them is critical to the development of inclusive therapies. Chronic injuries continue to represent the majority of extant cases in the absence of effective acute interventions, and pose a harder problem for clinical repair because of a diminution of injury-induced plasticity and progressive neuropathic changes.^{15,16} Cervical and upper thoracic injuries can cause profound visceral deficits in addition to severe sensory and motor disability.^{2,17} The cervical region is the most common region of spinal injury, constituting, for example, >50% of new SCI cases reported in recent years in the United States.¹⁸

We report here on four series of experiments, in which one or more autonomic deficits were studied. Elevation of intracarotid blood pressure and alterations in heart rate were produced by rectal distension or noxious mechanical stimulation of the paws under ketamine-xylazine anesthesia. Exaggerated pressor responses are a key sign of autonomic dysreflexia (AD).^{19–21} The slowing of gastric emptying (GE) was determined by dye recovery assay. In addition, serum insulin and glucose, along with other standard substances, were assayed by drawing blood at different times from the tail. In the first series, 1 week of unilateral PAG stimulation was started 1–2 h after the injury and was found to block the exacerbation of pressor responses measured on post-injury week 6. The second series examined older injuries, finding normal pressor responses, but an unexpected bradycardia on weeks 18–19, which was reversed by PAG stimulation started on post-injury week 7 or 12. In the third series, abnormalities in both GE and insulin level were present on weeks 14–15, and both were prevented by 5 weeks of PAG stimulation started in post-injury weeks 7–8. A final series confirmed that the NRM could serve as a relay for the PAG's spinal restorative effects. Both GE and excessive pressor responses in week 9 were normalized by stimulating the NRM for several weeks starting 2 days after injury. A histochemical correlate of PAG-produced functional recovery was sought caudal to the zone of damage, in thoracic segment T1, by immunostaining for calcitonin gene-related peptide (CGRP) in the superficial dorsal horn. CGRP is an important neurotransmitter of visceral primary afferents that increases in the thoracolumbar spinal cord after SCI.^{22–25} Our findings broadly strengthened the hypothesis that the descending PAG–NRM axis has restorative influences on spinal cord contusion, acting at both acute and chronic stages.

Methods

Surgical creation of injuries

All procedures on animals followed a protocol approved by the Institutional Animal Care and Use Committee of the University of Miami Miller, School of Medicine. Adult female Sprague–Dawley rats (weight 225–250 g) received injuries while anesthetized with isoflurane (1.5–2% in oxygen) delivered through a facemask. A heating blanket responding to a signal from a rectal thermistor maintained the body temperature at 37°C. A laminectomy revealed spinal cervical segment C5, and an Infinite Horizon Impactor device (Precision Systems & Instrumentation, LLC, Lexington, KY) produced a midline contusion injury with a set force of 200 or 250

kdyn and no dwell time, propelled at a velocity of 1 mm/sec with a contact diameter of 2.5 mm. These forces are known to produce mild (200 kdyn) or moderate (250 kdyn) injuries.²⁶

After injury, the dissected muscle layers were sutured and the skin was closed with wound clips. Animals recovered on a 37°C heating blanket. The opioid buprenorphine (0.01 mg/kg bid, subcutaneous) was given over 3 days for analgesia, and the antibiotic gentamycin (0.01 mg/kg b.i.d., subcutaneous) was given over 7 days to prevent infection. Bladder volume was checked daily; manual emptying was not necessary for more than 3 days, consistent with previous findings with this injury model.²⁶

Stimulator design and implantation

A self-powered wireless stimulator, described in detail elsewhere,²⁷ was implanted in the NRM or PAG. The stimulator was enclosed in a cuboid epoxy capsule, from which protruded a cathodal microelectrode and a bare stainless steel wire anode. Most capsules were 15 mm long, 8 mm wide, and 4 mm high. Some longer and taller capsules (18 mm long, 8 mm wide, 6 mm high) were used in one experimental series (series 3) to contain larger batteries and modified circuitry, which allowed longer lifetimes and variable pulse amplitudes (programmable up to 70 μ A in 10 μ A steps). The tungsten microelectrodes (AM Systems Inc., Sequim, WA; model 573210) were of 0.5 M Ω nominal impedance and shortened to reach the stereotaxic target of the NRM or PAG while leaving 1 mm clearance between the capsule and skull. All stimulators signaled their program status by emitting width-modulated infrared pulses detected by a handheld receiver box. Readings were taken at least once a day. Relevant parameters (clock time, pulse width or amplitude, active or inactive status) were controllable with magnetic pulses applied nearby.

Implantation of the stimulator and electrodes took place on the day of injury or at various later times. The rats were anesthetized with isoflurane as described, and their heads were positioned in a stereotaxic holder. A small hole ($\approx$ 1 mm diameter) was drilled in the dorsal surface of the skull for microelectrode access. Target coordinates with respect to the interaural line in the flat-skull plane for the NRM were 0 mm lateral, 2.3 mm caudal, and 0 mm dorsal, and for the PAG they were 0.7 mm lateral, 1.2 mm rostral, and 4 mm dorsal. Two small stainless steel screws were driven into the skull just rostral and caudal to the implanted capsule. The wire forming the anode was wrapped around the rostral screw to make a firm electrical contact. Screws, capsule and exposed electrode wires were covered in rapidly hardening poly-methyl-methacrylate cement that adhered to the skull. When the studied animals awoke from surgery, untoward acute reactions to the stimulation, such as overt twitches or aversive behavior, were absent.

Experimental series and groups

Four series of experiment were performed, in which injured rats were assigned randomly to stimulated or control (nonstimulated) groups. Series 1–3 targeted the PAG, and series 4 targeted the NRM. The programmed stimulation always consisted of 8 Hz trains of 1 ms rectangular cathodal pulses, automatically given daily over 12 daylight hours in 10 min cycles (5 min on, 5 min off), with no stimulation given for 12 night-time hours. The amplitude of the constant-current stimulus was 30 μ A in series 1, 2, and 4, and 70 μ A in series 3.

With regard to the timing of interventions, stimulation in series 1 and 4 was commenced within hours or days of the injury (acute injury), whereas in series 2 and 3, stimulation began $\geq$ 7 weeks after injury (chronic injury). Specifically, stimulation was started within a few hours of injury in series 1, 7–8 or 12 weeks after injury in series 2, 7 weeks after injury in series 3, and 1–2 days after injury in series 4. Weeks are numbered here so that week 1 starts on the day of injury (day 0). The planned duration of stimulation was 6 days in series 1, 3 weeks in series 2, 5 weeks in series 3, and 2.5 weeks in series 4. Euthanasia followed by histological analysis was

planned for 6 weeks after injury (series 1), 18–19 weeks after injury (series 2), 13 weeks after injury (series 3), and 8 weeks after injury (series 4). Control rats received identical injuries and surgeries, but their stimulators were left inactive. Additional groups received neither an injury nor a stimulator implant (intact rats), to provide normal data for variables that could only be measured once (usually terminally).

After stimulation for the designated number of days, devices were inactivated. When infrared signals could not be detected in daily readings, stimulation was assumed to have stopped as a result of battery failure, and stoppage was assured by applying the inactivating pattern of magnetic pulses. Animals were assigned to the stimulated group if the infrared signal disappeared more than halfway through the planned duration of treatment, and to the nonstimulated group if failure occurred within 24 h. Rats stimulated for other durations were excluded from further analysis. Measured times and injury forces, as opposed to planned values, are presented as means in Table 1, along with a listing of the outcome variables examined for each group.

Various weekly motor tests relevant to cervical injuries (locomotion on a catwalk, hanging by forelimbs, forelimb grip strength, and stability on an inclined plane) were conducted identically on treated rats and their controls. In addition, whole body plethysmography measurements of breathing were performed weekly. Findings from these tests will form the subject of a future article.

Blood pressure and heart rate measurements

Pressor responses indicative of AD were evaluated by a modified version of several published protocols.^{28,29} Rats were anesthetized with 50 mg/kg ketamine plus 10 mg/kg xylazine (both intraperitoneal). A cannula containing heparinized saline was inserted into a common carotid artery and was connected to a transducer for monitoring blood pressure (Stoelting Co., catalog number 50110); this device was calibrated before testing on each rat. The upper rectum and adjacent colon were distended for 100 sec by rapidly injecting a 4 mL volume of air into a balloon catheter. This procedure was repeated three to five times at intervals of ≥ 8 min (Fig. 1). To examine pressor responses to cutaneous noxious stimulation, a spring-loaded, serrated clip applied a constant-force (10 kdyn) to each paw in turn for a period of 1 min at intervals of ≥ 3 min. The peak in mean arterial pressure (MAP) that occurred either during the rectal or cutaneous stimulus or during the subsequent 1 min was subtracted from the resting value. In a small number of cases, the MAP declined monotonically, in which case

the nadir was similarly analyzed and included in group averages. The resting MAP or heart rate was calculated as an average over the 20 sec immediately before the start of noxious stimulation. Changes in heart rate were measured in 10 sec periods centered on the MAP peak (or nadir), and subtracted from the rate prevailing in the 10 sec before the noxious stimulus.

Gastric emptying

Gastrointestinal motility was assessed by slightly modified published protocols.^{30–32} After a 24 h fasting period, awake rats were fed by gavage a 1.5 mL volume of 0.5 mg/mL Phenol Red (Sigma, St. Louis, MO) in 5% glucose solution. Fifteen minutes later, an overdose of ketamine and xylazine (120 mg/kg + 20 mg/kg) was followed by surgical removal of the gastrointestinal tract via laparotomy. The stomach was clamped at the cardia and pylorus, and the terminal ileum was also clamped. The small intestine was divided into duodenum ($\sim 40\%$), jejunum ($\sim 30\%$), and ileum ($\sim 30\%$). The volumes of these four extracted tissues were measured by fluid displacement and then homogenized for 30 sec. After 20 min to allow for settling of solids, the fluid was centrifuged for 10 min at 2800 rpm. Proteins in 5 mL of the resulting supernatant were precipitated with 0.5 mL of trichloroacetic acid (20% w/v) and then centrifuged for 20 min at 2800 rpm. The final supernatant (3 mL) was added to 4 mL of 0.5 N NaOH. Samples (50 μ L) were plated in quadruplicate in a 96 well microplate, along with a dilution series (0.5–0.0002 mg/mL) to generate a standard linear curve. Absorbance at 560 nm was measured by a microplate spectrophotometer (SpectraMax M5, Molecular Devices, Sunnyvale, CA). The mass of the recovered phenol red was calculated from the absorbance, the dilution curve, and the original tissue volume.

Serum analysis

Serum was collected from each individual on week 0 (pre-injury), week 7 (pre-implantation), and week 14 (post-stimulation or control). The tip of the tail was first coated in local anesthetic cream, and then, ~ 3 min later, the tail was dipped in warm water (40°C) to cause vasodilation. A cut was made 0.1–0.2 cm from the tip of the tail using a sterile scalpel blade. Blood was collected from the wound, and then pressure was applied to stop the bleeding. The blood was allowed to clot at room temperature for 20 min. It was centrifuged at 4°C in a plastic gel serum separator Capiject Tubes (Terumo, 3T-MLHG) for 10 min at 2500 rpm. The serum portion was collected and quickly stored at -80°C . Serum insulin was assayed by an ELISA kit (ThermoFisher Scientific, catalog item

TABLE 1. EXPERIMENTAL GROUPS: THEIR TREATMENTS AND OUTCOME MEASURES

Series, group	n	Stim.	Target	Stim. start ^a (days ^b)	Stim. duration (days ^b)	Survival ^a (days ^b)	Contusion injury (force, kdyn ^b)	Outcome measures
1, Stim	10	Yes	PAG	0.1	7.0 $\pm$ 0.1	38.2 $\pm$ 0.2	210 $\pm$ 2.3	CV
1, Control	8	No	PAG	-	-	38.3 $\pm$ 1.5	206 $\pm$ 2.5	CV
2, Early	5	Yes	PAG	47.0 $\pm$ 1.3	22.4 $\pm$ 1.7	125.7 $\pm$ 1.6	208 $\pm$ 2.3	CV
2, Late	5	Yes	PAG	81.8 $\pm$ 1.6	23.0 $\pm$ 0.8	123.0 $\pm$ 1.8	209 $\pm$ 2.9	CV
2, Control	8	No	PAG	-	-	-	206 $\pm$ 2.6	CV
3, Stim	12	Yes	PAG	46.4 $\pm$ 1.0	34.7 $\pm$ 2.2	94.0 $\pm$ 0.2	263 $\pm$ 1.9	GE, serum
3, Control	10	No	PAG	-	-	98.9 $\pm$ 0.8	266 $\pm$ 3.5	GE, serum
4, Stim	11	Yes	NRM	1.5 $\pm$ 0.2	17.1 $\pm$ 1.5	57.6 $\pm$ 0.3	262 $\pm$ 1.4	GE, CV
4, Control	13	No	NRM	-	-	59.2 $\pm$ 0.7	262 $\pm$ 2.4	GE, CV

^aFrom time of injury.

^bMean $\pm$ s.e.m.

Stim, stimulation; PAG, periaqueductal gray; NRM, nucleus raphe magnus; CV, cardiovascular; GE, gastric emptying.

FIG. 1. Examples of pressor responses measured through a cannula in a common carotid artery during 100 sec of rectal distension. The output of the pressure transducer was digitized at 130 samples per second then plotted as a 250 point moving average covering 1.9 sec, which included five to eight cardiac cycles. Each graph shows three consecutive tests, plotted in blue (first test), red (second test), and green (third test). The purple horizontal bar between 50 and 150 sec indicates the period of distension. Representative animals from all tested injured groups, as detailed in Table 1, are shown.

ERINS), with quantification performed according to the manufacturer's instructions. A standard blood panel assay was provided by the veterinary clinical laboratory at the University of Miami, including the following substances of interest: glucose, blood urea nitrogen (BUN), creatinine (crea), amylase, calcium, phosphorus, total protein, albumin, aspartate transaminase (AST), aspartate aminotransferase (ALT), creatine phosphokinase (CPK), and total bilirubin.

Histology

Animals were euthanatized with intraperitoneal ketamine (120 mg/kg) and xylazine (dosage 20 mg/kg) and perfused transcardially with 4% paraformaldehyde. Spinal cords were carefully extracted, post-fixed overnight in paraformaldehyde, and embedded in paraffin. A length of spinal cord, 2.5–7.5 mm below the injury, was cut in coronal sections (12 μ m). Some sections were stained with hematoxylin and eosin. Others were immunostained with anti-CGRP (Calbiochem, Darmstadt, Germany). Specimens were incubated overnight at 4°C with the primary anti-CGRP antibody (1:200) and then incubated at room temperature for 2.5 h with secondary donkey anti-rabbit antibody (Alexa Fluor 488, 1:250, Invitrogen, Carlsbad, CA). For triple labeling, anti-NeuN (1:500, MAB377 Millipore) was present with the anti-CGRP, and

revealed by donkey anti-mouse antibody (Alexa Fluor 594, 1:250, Invitrogen), and 4',6-diamidino-2-phenylindole (DAPI) (Vector Labs) was included in the mounting medium. Images of CGRP staining were acquired with a UPLSAPI 10 $\times$ NA 0.40 objective on an Olympus FV1000 confocal microscope using identical image-acquisition parameters. Three serial sections 240 μ m apart from approximately the first thoracic segment were analyzed for color thresholds with the open-source platform Fiji.³³ CGRP expression was quantified in laminae I and II of the dorsal horn in terms of staining density, after subtraction of background density measured in a 50 $\times$ 50 μ m area of tissue with no immunoexpression.

Statistical analysis

Statistical analysis employed the SPSS software package (v. 24, IBM), applying a significance level of $p < 0.05$ in two sided tests. For the cardiovascular variables, gastric emptying and CGRP density, a one way ANOVA was conducted within each tested series on nonstimulated (injured), stimulated (injured), and intact groups. The ANOVA was followed by post-hoc comparison of the intact and stimulated groups against the nonstimulated group using Dunnett's test. Each substance assayed in serum was subjected to one way univariate ANOVA, followed by repeated contrast (k-

matrix) analysis of consecutive groups: week 0, week 7, week 14 nonstimulated, and week 14 stimulated. The observed power ($1-\beta$) was calculated from the type II error rate (β) for $\alpha=0.05$, along with the effect size (partial η^2). Detailed statistical results with ANOVA are presented in the figures and their legends. Mortality rates were obtained by the Kaplan–Meier method.

Results

Mortality and injury force

The injuries created with the nominal 250 kdyn force were measured on impact to be ~ 260 kdyn (Table 1) and were associated with an acute 24 h mortality rate of $0.17 (\pm 0.13, \text{binomial } 95\% \text{ confidence interval})$. The less severe, nominally 200 kdyn injuries, measured to be ~ 205 kdyn, produced an acute 24 h mortality rate of $0.083 (\pm 0.07 \text{ confidence interval})$. Deaths after the first 24 h were infrequent and of uncertain cause. Examples of the histology and morphology of injured tissue are given in Figure 2. Informal examination suggested that the lesion size was not systematically affected by the different treatments.

Blood pressure and heart rate

The MAP increased in most tests of rectal distension or squeezing of the forepaws or hindpaws. The rise was sometimes biphasic, with an initial decrease (Fig. 1). A monophasic fall was seen in 3% of all tests (10 of 348), more frequently at weeks 17–18 (8% of tests, 7 of 95) and with rectal distension (5%) than with paw pressure ($< 1\%$). In series 1, PAG stimulation was applied for 1 week in acute injury. Without this treatment, the peak pressor response to rectal distension, measured in the 6th post-injury week, was significantly higher on average in injured than intact rats (Fig. 3A). The early PAG stimulation significantly reduced this untreated pressor response to approximately that of intact rats. The peak in MAP during pinching of

the forepaws but not the hindpaws was also significantly greater in untreated rats (Fig. 3A), rising about twice as in rats that were given PAG stimulation or were never injured. In the study of PAG stimulation in chronic SCI (series 2), injured untreated rats in weeks 18–19 post-injury showed no difference from intact rats in pressor responses elicited by rectal distension or by squeezing of the forepaws or hindpaws. An effect of PAG stimulation on pressor responses was, therefore, not expected in this series, and was absent in both the early subgroup (starting on week 7) and the late subgroup (starting on week 12). The two subgroups were combined for purposes of display and analysis (Figs. 3 and 4). NRM stimulation for 17 days, starting 1–2 days after injury (series 4), mirrored the effect of acute PAG stimulation. The pressor response to rectal distension, measured in week 9, was significantly reduced compared with nonstimulated rats, and was near the level of intact animals (Fig. 3C). However, unlike the 200 kdyn injuries in series 1, the untreated 250 kdyn injuries in series 4 showed no significant change in pressor responses to pinching of the forepaws or hindpaws compared with intact rats, and NRM stimulation did not significantly affect these responses to pinching.

Evoked changes in heart rate in rats in early weeks after untreated injury showed no difference from intact rats (series 1 and 4), including in their response to rectal distension or nociception of the forepaws or hindpaws. There were, further, no significant effects of acute PAG or NRM stimulation on these changes (Fig. 3D, F). By contrast, in rats with older untreated injuries (series 2), heart rate was significantly lower than normal during rectal distension (Fig. 3E) and hindpaw pinching, but not during forepaw pinching. This bradycardic response was abolished by stimulating the PAG for 3 weeks, starting 7 or 12 weeks after injury (Fig. 3E).

Resting levels of MAP or heart rate, in most comparisons, were not significantly altered by injury in prior weeks or by subsequent PAG or NRM stimulation (Fig. 4). An exception was heart rate in rats with older untreated injuries (18–19 weeks, series 2), which

FIG. 2. Spinal cord lesions. (A) Dorsal view of freshly removed cervical spinal cord on a brown background, 17 weeks after a 200 kdyn (nominal) injury. The rat was stimulated for 3 weeks starting in week 6 post-injury. A gray zone of injury-induced demyelination is indicated by the unfilled arrow. Three cervical dorsal roots (4–6) are labeled. (B–E) Histological sections stained with hematoxylin-eosin and co-stained with Luxol fast blue, to show myelination. Dorsal is upwards. The 0.5 mm scale bar (bottom right) applies to B–E but not to A. The main injury cavity is marked by an asterisk. B is a sagittal section through the midpoint of same rat depicted in A; the filled arrow points in the rostral direction. C is a coronal section through the midpoint of a nominal 250 kdyn injury; this rat was never stimulated. D is a coronal section through the midpoint of a nominal 200 kdyn injury; this rat was stimulated for 6 days starting on the day of injury. E is from the mid-cervical spinal cord of an uninjured rat, for comparison.

FIG. 3. Peak changes in mean arterial pressure (MAP) and heart rate during prolonged rectal distension (“rectum”) or prolonged squeezing of the forepaws or hindpaws in intact rats, injured controls (“no stim.”), and stimulated rats (“stim.”). Top row: (**A**) effects on MAP of early periaqueductal gray (PAG) stimulation (series 1), (**B**) late PAG stimulation (series 2), and (**C**) early nucleus raphe magnus (NRM) stimulation (series 4). Bottom row: corresponding effects on heart rate. Post-hoc comparisons used the Dunnett test testing against the group that received no stimulation. All significant post-hoc tests ($p < 0.05$) are indicated by the square brackets along with p values rounded to three decimal places. Values from significant main ANOVA were as follows: **A**, rectum $F_{2,25} = 6.53$, $p = 0.005$, effect size 0.343, $(1-\beta) = 0.87$; **A**, forepaws, $F_{2,22} = 5.17$, $p = 0.014$, effect size 0.32, $(1-\beta) = 0.77$; **C**, rectum, $F_{2,28} = 3.80$, $p = 0.035$, effect size 0.21, $(1-\beta) = 0.64$; **E**, rectum, $F_{2,25} = 13.02$, $p = 0.0001$, effect size 0.51, $(1-\beta) = 0.99$; **E**, forepaws, $F_{2,24} = 7.98$, $p = 0.002$, effect size 0.40, $(1-\beta) = 0.93$. The group sizes are for the intact group (all graphs) 10–11, early PAG stimulation 7–9, early PAG control 7–8, late PAG stimulation 9–10, late PAG control 6–7, NRM stimulation 6–8, NRM control, 13–14.

was significantly lower than normal in the resting condition (Fig. 4E), paralleling the bradycardic response observed during rectal distension and hindpaw pinching (Fig. 3E). Treatment with 3 weeks of PAG stimulation starting 7 or 12 weeks after injury restored the mean resting heart rate to approximately that of uninjured animals (Fig. 4E).

Gastric emptying

Two experimental series examined GE: one testing acute intervention with NRM stimulation (series 4) the other testing chronic intervention with PAG stimulation (series 3). In both series, a higher injury force was used (250 kdyn), to increase the likelihood of a

FIG. 4. Resting mean arterial pressure (MAP) and heart rate displayed and analyzed as in Figure 3. Top row: (A) effects on resting MAP of early periaqueductal gray (PAG) stimulation (series 1), (B) late PAG stimulation (series 2), and (C) early NRM stimulation (series 4). Bottom row: corresponding effects on heart rate. Values from the significant main ANOVA in E were as follows: $F_{2,25} = 8.83$, $p = 0.001$, effect size 0.41, $(1-\beta) = 0.95$. Group sizes are described in the legend for Figure 3.

deficit. In series 3, to increase the likelihood of significant restoration, PAG stimulation was given for a longer period and at a higher amplitude. The quantity of phenol red marker remaining 15 min after administration was determined for four compartments: the stomach and three lengths of small intestine, corresponding to the duodenum, jejunum, and ileum. The total amount of recovered dye was assumed to add to 100% of the amount administered. Results were plotted and analyzed as the percentage of total dye that had passed from each of the first three compartments to the next one.

NRM stimulation, which was given for 17 days starting 1–2 days after injury, restored GE to near normal levels on week 9 after injury (Fig. 5A). Elimination from both the stomach and the duodenum was significantly more rapid in intact and stimulated rats than in injured controls. The difference between intact and injured control groups extended to the jejunum, although this was not significantly influenced by the stimulation. These results were replicated in most details when PAG stimulation was started on weeks 7–8 after injury and given for 5 weeks, with GE assessed in weeks 14–15 after injury. In

FIG. 5. Emptying of stomach and small intestine after (A) early nucleus raphe magnus (NRM) stimulation (series 4) and (B) late periaqueductal gray (PAG) stimulation (series 3). All injuries employed a nominal 250 kdyn force. Significance in Dunnett post-hoc comparisons ($p < 0.05$) is indicated by p values next to square brackets. Findings of the main ANOVA were as follows: **A**, stomach, $F_{2,22} = 4.17$, $p = 0.031$, effect size 0.31, $(1-\beta) = 0.66$; **A**, duodenum, $F_{2,22} = 5.18$, $p = 0.016$, effect size 0.35, $(1-\beta) = 0.76$; **A**, jejunum, $F_{2,22} = 4.68$, $p = 0.022$ effect size 0.33, $(1-\beta) = 0.72$; **B**, stomach $F_{2,29} = 16.04$, $p = 0.0002$ effect size 0.53, $(1-\beta) = 1.00$; **B**, duodenum, $F_{2,29} = 14.34$, $p = 0.0005$, effect size 0.50, $(1-\beta) = 1.00$; **B**, middle jejunum, $F_{2,29} = 9.43$, $p = 0.007$, effect size 0.39, $(1-\beta) = 0.97$. The data for intact group are the same in the two graphs.

nonstimulated controls with PAG electrodes, the stomach, duodenum, and jejunum again showed significant effects of injury. PAG stimulation significantly improved the emptying of the stomach and duodenum (Fig. 5B), and, unlike NRM stimulation, improved the emptying of the jejunum as well.

Serum chemistry

Series 3 measured serum chemistry at three time points (Fig. 6): 1 day before injury, in the 7th week after injury (just before implantation of stimulators) and in the 14th week after injury (1 week after the end of stimulation, if given). The focus was on insulin and glucose, although we also report here the concentrations of other substances yielded by the standard blood chemistry panel. Insulin was unaffected by injury at 7 weeks, but showed a large decline on week 14. This decline was reversed significantly by the PAG stimulation. Glucose appeared to be tightly regulated after injury, with no significant change in untreated rats at 7 or 14 weeks post-

injury, but PAG stimulation, which produced a significant fall in glucose, concomitant with the recovery of insulin.

With regard to other substances (Fig. 6), significant declines occurred in two substances on week 7, phosphorus and albumin, which were maintained on week 14. On week 14, both BUN and crea were significantly higher than on week 7, although their ratio was unaffected, whereas amylase and calcium were significantly lower. Apart from glucose and insulin, only CPK (a marker of muscle atrophy) was significantly affected by the prior PAG stimulation, falling back to below the pre-injury level.

Histological analysis

Staining for CGRP was mainly observed in the dorsal half of the spinal cord (Fig. 7B). Its density was quantified in laminae I and II, caudal to the center of the lesion at approximately segment T1. All series that used PAG stimulation were analyzed. Injury led to an increase in CGRP at all three post-injury stages studied: 6 weeks, 14–15 weeks, and 18–19 weeks (Fig. 7A). In rats with 200 kdyn

FIG. 6. Blood levels of insulin measured by ELISA and of glucose and other substances obtained by a standard laboratory blood panel. The bars are coded as follows: black, intact (never stimulated); white, injured and never stimulated; gray, injured and stimulated (“stim.”). Units for the vertical axes are given in the titles. Week 0 is before injury; week 7 is just before device implantation and 7 weeks after injury; week 14 was 2 weeks after ~5 weeks of stimulation had been given to the stimulated group. Depending on the substance, the groups have the following numbers of individuals: week 0, 8–10; week 7, 8–11; week 14 (no stim), 8–11; week 14 (stim), 10–11. Significance in a repeated contrast analysis of the k-matrix is indicated by the horizontal brackets associated with p values. Significant contrasts from the main ANOVA (one way, univariate) were as follows: insulin, $F_{3,36}=5.71$, $p=0.003$, effect size 0.32, $(1-\beta)=0.92$; glucose, $F_{3,39}=7.83$, $p=0.0002$, effect size 0.38, $(1-\beta)=0.98$; blood urea nitrogen (BUN), $F_{3,39}=3.56$, $p=0.022$, effect size 0.21, $(1-\beta)=0.75$; creatinine (crea), $F_{3,39}=9.04$, $p=0.0002$, effect size 0.41, $(1-\beta)=0.99$; calcium, $F_{3,39}=8.22$, $p=0.0004$, effect size 0.39, $(1-\beta)=0.99$; phosphorus, $F_{3,39}=4.89$, $p=0.006$, effect size 0.27, $(1-\beta)=0.88$; albumin, $F_{3,39}=2.61$, $p=0.065$, effect size 0.17, $(1-\beta)=0.60$; creatine phosphokinase (CPK), $F_{3,34}=6.68$, $p=0.001$, effect size 0.37, $(1-\beta)=0.96$; amylase, $F_{3,38}=17.99$, $p=0.0001$, effect size 0.59, $(1-\beta)=1.00$.

FIG. 7. Staining density of calcitonin gene-related peptide (CGRP) in laminae I and II of the dorsal horn gray matter (first thoracic segment) after periaqueductal gray (PAG) stimulation ("stim.") compared with control rats with spinal cord injury (SCI) ("no stim.") and intact rats. **(A)** Measurements from the three experimental series. Values of p , derived from Dunnett post-hoc comparisons, are given next to the bars over each pair analyzed. Staining density is normalized to the intact value. Group numbers were as follows: for series 1, no stim. $n=4$, stim. $n=4$, and intact $n=7$; for series 2, no stim. $n=6$, stim. $n=8$, and intact $n=7$; for series 3, no stim. $n=9$, stim. $n=10$, and intact $n=7$. Values from intact animals for comparing acute and chronic 200 kdyn injuries (series 1 and 2) are from the same data set; a different set was used with series 3. The main ANOVA gave the following results: acute PAG stimulation (200 kdyn), $F_{2,12}=16.54$, $p=0.0004$, effect size 0.73, $(1-\beta)=0.99$; later PAG stimulation (200 kdyn), $F_{2,18}=12.01$, $p=0.0005$, effect size 0.73, $(1-\beta)=0.99$; later PAG stimulation (250 kdyn), $F_{2,23}=6.65$, $p=0.005$, effect size 0.37, $(1-\beta)=0.87$. **(B)** Representative examples of staining for CGRP in the dorsal horn, caudal to the C5 lesion in segment T1. The rightmost sections are triple labeled for CGRP (green), NeuN (red), and 4',6-diamidino-2-phenylindole (DAPI) blue, with laminae outlined and labeled (laminae I are II combined); dr = dorsal rootlet. Other sections show only CGRP, with the boundaries of laminae I-IV outlined and the spinal cord surface also demarcated.

injuries that had received PAG stimulation acutely (series 1), average staining density for CGRP was lower than in rats without treatment, approaching the intact level. Stimulation started in later weeks produced a similar reversal, both in series 2 (200 kdyn force) and series 3 (250 kdyn force). Scattered large fiber bundles were noted to be more common in lamina IV in untreated than in stimulated rats (Fig. 7B), but were not included in the quantification.

Discussion

General conclusions and experimental design

The present findings support the concept of the descending PAG–NRM–spinal axis as a restorative system that enhances re-

covery from SCI. Long-term autonomic deficits are shown for the first time to be reversible by interim brainstem stimulation, including deficits of more than 4 months' standing in bradycardia, GE, and insulin level. These results complement prior studies, in which NRM or PAG stimulation, given in the 1st week of incomplete thoracic SCI, led to improved recovery of sensory-motor performance.^{4,5} The PAG's direct projection to the spinal cord is minor in rats; therefore, its influence on recovery after SCI is probably relayed by its strong excitatory input to the NRM.^{9–12} A conceivable alternative relay to the spinal cord is through the rostral ventrolateral medulla (RVLM), which mediates the immediate cardiovascular effects of the PAG.³⁴ The long-term trophic effects of the RVLM are unknown, however, whereas the NRM has clear

benefits after neurotrauma and releases trophic substances (serotonin, several neuropeptides) as will be discussed.

Within the experimental paradigm of interim stimulation for SCI, countless variations are possible. The spinal location of the injured tissue, the means of producing the injury (e.g., mechanical or chemical), the site of stimulation, and histochemical and functional outcome measures can all be varied. The stimulation can start and stop at various times after injury; use trains of different frequency, amplitude, and pulse width; and be cycled on and off at different frequencies, duty cycles, and diurnal periods. Systematic sampling of all these parameters is prohibitive, although many are of possible interest and importance. We approached this problem by making stepwise adjustments so that the accumulating results met the main experimental goals of reversing acute and chronic autonomic deficits. Some combinations were necessarily neglected, such as the NRM's effects in chronic SCI and the effects of PAG stimulation on GE and serum insulin in acute SCI.

Central control of repair

A large experimental literature describes the electrophysiological responses of raphe neurons to various sensory and chemical stimuli, their anatomical projections, and the substances (serotonin and neuropeptides) released by many of their terminals. In summary, this literature indicates a highly divergent axonal system that fires in response to sensory and chemical signals that are generally associated with trauma, such as pain, hypotension, hypercapnia, hypoxia, hypothermia, and circulating cytokines.^{12,35–39} Serotonin has well-documented trophic influences.^{40,41} We previously reported that 2 h of NRM stimulation on the 3rd day after T8 contusion restored the major trophic intracellular signaling molecule cyclic adenosine monophosphate (cAMP) in all spinal segments, an effect that was blocked by the 5-HT₇ receptor antagonist pimo- zide.⁷ In the same study, there was increased phosphorylation (activation) of trophic molecules downstream to cAMP, namely protein kinase A (PKA) and cAMP response element-binding protein (CREB). In a parallel study, the median raphe nucleus was found to restore forebrain levels of cAMP after traumatic brain injury.⁶ Neuropeptides released by the raphe system include thyrotropin-releasing hormone, galanin, met-enkephalin, and substance P.^{42–44} Thyrotropin-releasing hormone has shown restorative effects after SCI.⁴⁵ Galanin is trophic and anti-inflammatory in traumatic brain injury and experimental allergic encephalitis (EAE).⁴⁶ Met-enkephalin has neuroprotective and anti-inflammatory actions.⁴⁷ Substance P has an ambivalent mixture of effects, not only promoting proliferation of spinal neural stem cells,⁴⁸ but also mediating inflammation after injury,⁴⁹ which may be beneficial in some phases or recovery.

A major central nervous system (CNS) injury thus leads inevitably to widespread release of beneficial molecules from terminals of excited raphe neurons. We suggest that this feedback controls many biochemical processes with effects on repair. Because we found functional and anatomical improvements when brainstem stimulation was initiated ≥ 6 weeks after injury, the injured tissue apparently retains a capacity to respond to restorative inputs far beyond the acute injury phase. This phenomenon is of potential clinical importance, although the selective advantage in wild populations is probably limited to recent injuries that are mild or occult.

Restorative effects on circulatory parameters

The autonomic nervous system can show various types of anatomical damage after a cervical contusion. This includes damage to

descending inputs (excitatory or inhibitory) to sympathetic pre-ganglionic neurons in segments C8–L2, the central portion of visceral afferents and pathways passing through cervical segments to sacral pre-ganglionic parasympathetic neurons.^{17,50} The increased pressor responses of AD probably results from increased sympathetic activity, whereas reduced GE and depressed insulin can be caused by greater sympathetic activity or less parasympathetic activity. Our observations on untreated injuries therefore revealed a general imbalance of sympathetic over parasympathetic activity that was most pronounced in earlier weeks. Not all autonomic signs of upper SCI follow this simple pattern; for example, orthostatic hypotension is common, and probably a result of partial sympathectomy.⁵¹

In humans, excessive pressor responses and reflex bradycardia, along with various other signs and symptoms including severe headaches and extreme sweating, constitute the syndrome of AD, all of which can reflect sympathetic hyperactivity.^{19–21} Several mechanisms have been proposed for these exacerbated pressor responses,⁵² based on cellular changes observed in different AD models. These include loss of descending serotonergic axons that inhibit nociceptive reflexes⁵³; plasticity of central afferent arbors, including those containing CGRP, induced by nerve-growth factor (NGF)⁵⁰; secondary changes of diminished vagal sensory processing from the gastrointestinal system⁵⁴; and greater adrenergic sensitivity of vascular smooth muscle to nerve stimulation.⁵⁵

Both the nociceptive (input) and the sympathetic (output) component of the measured pressor responses could have been affected by the prolonged brainstem stimulation. Anti-nociception is consistent with our previous findings that prolonged NRM stimulation in acute thoracic injuries increased serotonergic innervation in the injured spinal cord⁴ and suppressed allodynia.⁵ Activation of the PAG is known to stimulate the local release of the endogenous opioid β -endorphin,⁵⁶ but all released substances probably disappear completely in the interval between the end of stimulation and autonomic testing (13–56 days, across all groups). It is possible that β -endorphin is involved in other ways, however. Its release could contribute to functional recovery from SCI, based on the increasing evidence for plastic effects of μ -opioids.⁵⁷ Another unexplored possibility is that the brainstem stimulation produces long-term changes in opioid expression that oppose hyperalgesia and allodynia after SCI. These questions remain to be pursued by neurochemical assay and by pharmacological analysis of the relevant regions.

In rat models, blood pressure changes in AD have typically been studied in awake animals of the Wistar strain, with thoracic injuries made by transection or clip compression.⁵⁸ The Sprague–Dawley rat strain, used here for consistency with our previous work on brainstem stimulation in SCI,^{4,5} is reported to have a smaller pressor response to colorectal distension after SCI.⁵⁹ Thoracic injury sites, unlike cervical ones, involve direct changes in sympathetic pre-ganglionic neurons and their local interneuronal inputs.^{23,60,61} It was, therefore, not surprising that peak pressor responses in anesthetized Sprague–Dawley rats with mid-cervical contusion injuries at 6–9 weeks were approximately half of what is usually reported for other rat models and for episodic AD in human SCI.^{19,50} In contrast to the absolute change, the fractional effect was strong for both early PAG and NRM stimulation, reducing the evoked peak pressor response to 40% or 26% (respectively) of the untreated response. Differences in the injury model may also explain why injury did not significantly affect the MAP in resting conditions, when presumably the sensory factors involved in the evoked response are not as influential. A further indication that different factors are involved in resting MAP and evoked pressor responses after injury comes from published

observations that thoracic transection or clip compression injury causes lower resting MAP in awake rats with AD, while elevating the evoked pressor response.^{53,59}

At the post-injury time of 18–19 weeks, there was no significant difference in pressor responses between intact rats and those with injury (untreated or treated). This time period is at least twice as long as that used in other reported animal studies of AD,⁵⁸ although human SCI patients can manifest AD for years after injury.²⁰ The 200 kdyn injury force used for this series may have been too weak to produce permanent or long-lasting AD, given the previously reported dependence of AD on force in clip injuries.²⁵ A possible mechanism for normal pressor responses at weeks 18–19 after injury, as opposed to their elevation at weeks 6 and 9, is the strong recovery that descending serotonergic pathways exhibit in rodents after SCI,^{62,63} leading to improved pain control. Plastic mechanisms for gradual recovery of cardiovascular control have likewise been uncovered by a study of renal sympathetic nerve activity in rats with thoracic (T3) left hemisections, which found baroreflex control to be impaired 1 week after injury and to have recovered by 8 weeks.⁶⁴

The observation of significant resting and evoked bradycardia at post-injury weeks 18–19 but not weeks 6 or 9 was unexpected, and may also be a function of the cervical injury model and the anesthetized preparation. Here we can only offer tentative explanations that require further experimental verification. The evoked bradycardia presumably involves an exaggerated baroreceptor reflex evoked by the (normal) rise in MAP, which could be a consequence of abnormal growth of central connections or peripheral hypersensitivity. As regards the resting bradycardia, SCI is generally associated with reductions in mean cardiac output (Q) and stroke volume (SV),^{65–67} most likely driven by the lessening of metabolic demand, but also a possible consequence of deficient venous return or, in upper level injuries, a direct effect of sympathetic damage. Additionally, there are increases in vascular stiffness in chronic cervical SCI,⁶⁸ which imply a lower Q if the recovered baroreflex is to maintain normotension. Although Q can be largely reduced by a lower SV, bradycardia may come into play in older untreated injuries; for example, as an adaptive response to achieve energetically efficient pumping after sympathetic control of SV has partially returned.

Changes in gastric slowing and serum chemistry

Both insulin release from pancreatic islet cells and gastric emptying are facilitated by parasympathetic (vagal) activity and depressed by sympathetic activity. Both were found to be depressed 14 weeks post-injury in the present study. Slower GE is a typical deficit of upper level SCI in rats, although these deficits are reported to be absent after 4–6 weeks or in thoracic contusions.^{30,69,70} In humans, GE can remain impaired many years after injury.⁷¹ In contrast to AD, GE has a strongly facilitating parasympathetic motor component as well as a vagal sensory component (filling). The ascending vagal effect on GE has been found to be compromised after thoracic (T3) SCI, whereas sympathetic outflow to the stomach is unaltered.⁷²

Serum insulin is reportedly lower after chronic (16 week) thoracic (T3) transection in rats, with stable glucose levels but increased glucose tolerance compared with intact controls.⁷³ Insulin resistance is also observed in humans with chronic SCI.⁷⁴ Similarly, we found unchanged fasting glucose on week 14 in untreated rats with C5 SCI, even in the presence of lower basal insulin. The fall in glucose on week 14 after PAG stimulation, in association with recovery of the pre-injury insulin level, suggests a return of

insulin sensitivity. In addition to insulin and glucose, the only other substance significantly affected by the prior weeks of PAG stimulation was CPK, whose lower mean level may reflect less ongoing muscle degeneration.⁷⁵ Injury itself produced decreases in phosphorus and albumin on week 7 that remained on week 14, perhaps because of underlying renal problems. Several other substances were changed from pre-injury levels on week 14 but not week 7, including calcium, insulin, BUN, creatinine, and amylase, possibly stemming from increases in catabolism, renal problems, and bone resorption. In sum, a distinct serum signature was present in chronic SCI, but not all substances proved responsive to PAG stimulation.

Histological changes

In a previous study, we found that NRM stimulation had no significant effect on the size of the lesion cavity.⁵ It apparently improved functional connectivity in surviving tissue without producing major regrowth within the lesion. We therefore did not analyze lesion size in the present study, but focused on CGRP, which is elevated in thoracic and lumbar segments after thoracic SCI.^{23–25,50,76} Its elevation in lumbar but not thoracic segments is correlated with AD,²² strongly implying that increased sprouting of visceral nociceptive afferents is a major contributor to pelvically induced AD. However, the reversal by brainstem stimulation of increased pressor responses evoked by forepaw pinching suggests that visceral afferent sprouting in lumbar segments is not the only way that pressor responses are affected by the stimulation. It remains to be determined whether other neurotransmitters associated with nociceptive afferents, such as substance P, glutamate, adenosine triphosphate (ATP), and isolectin B4,^{77,78} also increase in superficial dorsal laminae at various segmental levels after injury and are susceptible to trophic brainstem control. The present findings on upper thoracic CGRP are probably more relevant to GE than to AD. CGRP is found in peripheral endings in the upper gastrointestinal mucosa and circular muscle as well as in central afferent terminals.⁷⁹ Experiments with a CGRP receptor antagonist in mice have shown that CGRP contributes to intestinal inflammation and postoperative ileus.⁸⁰ Hence, changes in CGRP are implicated in the effects of injury and of PAG and NRM stimulation on GE.

Clinical implications

Interim DBS of the PAG holds the possibility of a safe treatment for SCI, which requires little or no technical development.⁸¹ The NRM, although the probable origin of the restorative direct spinal projection and perhaps the more effective target, is more problematical from the standpoint of surgical safety. For example, it risks disturbing nearby hindbrain centers that control vital functions, despite the apparent absence of critical safety issues seen with NRM stimulation in the present and prior experiments.⁴ PAG stimulation has been performed safely in many human patients.^{13,14} For the large population of patients with chronic incomplete SCI, as well as for those with subacute SCI, prolonged PAG stimulation, therefore, seems to offer a promising option for gaining some significant degree of clinical or histological improvement, and its principal drawbacks are common to all uses of invasive DBS.

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