The Impact of Midcervical Contusion Injury on Diaphragm Muscle Function

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Abstract

Midcervical contusion injuries disrupt descending ipsilateral excitatory bulbospinal projections to phrenic motoneurons, compromising ventilation. We hypothesized that a unilateral contusion injury at C3 versus C5 would differentially impact phrenic activity reflecting more prominent disruption of ipsilateral descending excitatory drive to more caudal segments of the phrenic motor pool with more cranial injuries. Phrenic motoneuron counts and evidence of diaphragm muscle denervation at individual neuromuscular junctions (NMJ) were evaluated at 14 days post-injury after unilateral contusion injury (100 kDynes). Whole body plethysmography and chronic diaphragm EMG were measured before the injury and at 3, 7, and 14 days post-injury. Contusion injuries at either level resulted in a similarly sized cavity. C3 contusion resulted in loss of $39 \pm 13\%$ of ipsilateral phrenic motoneurons compared with $13 \pm 21\%$ after C5 contusion ($p=0.003$). Cervical contusion injuries resulted in diaphragm muscle denervation (C3 contusion: $17 \pm 4\%$; C5 contusion: $7 \pm 4\%$; $p=0.047$). The pattern of denervation revealed segmental innervation of the diaphragm muscle, with greater denervation ventrally after C3 contusion and dorsally after C5 contusion. Overall, diaphragm root mean square electromyography activity did not change ipsilaterally after C3 or C5 contusion, but increased contralaterally ($\sim 11\%$) after C3 contusion only on the first day post-injury ($p=0.026$). Similarly, there were no significant changes in breathing parameters during eupnea or exposure to hypoxia (10% O₂)–hypercapnia (5% CO₂) at any time post-injury. Unilateral midcervical contusions minimally impair ventilatory behaviors despite phrenic motoneuron loss and diaphragm muscle denervation.

Key words: level of injury; neuromuscular junction; phrenic motoneuron; spinal cord injury; ventilation

Introduction

RESPIRATORY COMPLICATIONS ARE COMMON after cervical spinal cord injury (SCI) and result in substantial morbidity and mortality.^{1–3} Lesions at the midcervical region disrupt descending excitatory projections from premotor neurons located predominantly ipsilaterally in the medulla.^{4–6} In addition, contusion injury may result in direct damage to the phrenic motoneuron pool located between the C3 to C5 segments in humans⁷ and rats.^{8,9}

The level of injury is an important predictor of functional outcomes in patients with SCI, and differences in clinically meaningful outcomes are evident with lesions separated by two midcervical spinal cord segments.^{10,11} Unilateral midcervical contusion injuries at various levels comprising the phrenic motor nucleus will likely differ depending on the disruption of descending ipsilateral excitatory bulbospinal projections such that more cranial injuries also compromise projections to more caudal segments of the motor pool.

Animal studies of traumatic SCI commonly employ cervical contusion models,^{12,13} including unilateral lesions.^{14–17} Previous studies suggest moderate functional impairment^{12–14,18} and sig-

nificant phrenic motoneuron loss^{15–17} after midcervical contusion. The diaphragm muscle, however, displays a large reserve capacity for force generation^{19–22} with minimal functional impairment evident immediately after unilateral phrenic denervation except during higher force, nonventilatory behaviors (sighs, airway occlusion, and sneeze).²³

We hypothesized that a unilateral contusion injury at C3 versus C5 would differentially impact phrenic (diaphragm electromyography [EMG]) activity reflecting more prominent disruption of ipsilateral descending excitatory drive to more caudal segments of the phrenic motor pool with more cranial injuries (C3 contusion), with minimal evidence of impaired ventilatory function.

Methods

Experimental animals

Adult male Sprague-Dawley rats (280–300 g) were obtained from Harlan (Harlan Laboratories Inc, Indianapolis, IN) and were housed in a controlled environment with access to food and water. All procedures were conducted in accordance with the American

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Physiological Society Animal Care Guidelines and were approved by the Institutional Animal Care and Use Committee.

Animals were anesthetized with a mixture of ketamine (90 mg/kg) and xylazine (10 mg/kg) via intramuscular injection for all surgical procedures. A total of 32 male rats were randomly assigned to one of three groups: laminectomy ($n = 12$), unilateral C3 contusion ($n = 10$), and unilateral C5 contusion ($n = 10$). An additional three animals were included as an intact nonsurgical sham group for assessment of diaphragm muscle denervation as described below.

Diaphragm EMG recordings

Based on a previously described technique,^{22–33} two pairs of electrodes were implanted into each side of the diaphragm muscle 3–4 days before the contusion or laminectomy surgery. Electrodes were made of insulated stainless steel fine wire (AS631, Cooner Wire Inc., Chatsworth, CA) with ~ 3 mm of insulation stripped off for contact with the muscle. Each electrode pair was fixed into the midcostal diaphragm muscle with an interelectrode distance of ~ 3 mm and connected to an implanted telemeter (TR50BB, Millar Inc., Houston, TX), which was secured to the inner face of the anterior abdominal wall using a soft surgical mesh. Telemeters were sterilized using a glutaraldehyde-based solution as recommended by the manufacturer.

Rhythmic diaphragm EMG activity was recorded daily by telemetry in awake, unrestrained animals. Animals were left inside their cages and acclimatized for ~ 10 min before a 10-min recording. The EMG signal was amplified and band-pass filtered using LabChart 5 software (AD Instruments). To assess and quantify the amplitude of diaphragm EMG activity, root-mean-square (RMS) EMG signal was calculated.

Diaphragm EMG activity was independently measured for eupneic breathing and all spontaneous deep breaths (“sighs”) during the 10 min recording period. For both eupnea and sigh, diaphragm RMS EMG amplitude at 0, 1, 3, 7, and 14 days post-injury (DPI) was normalized to the sigh value at 0 DPI for the same diaphragm side and animal.

Unilateral midcervical spinal contusion

Anesthetized animals were placed on a heating pad (model 1060, K&H Manufacturing, Colorado Springs, CO). Using sterile technique and surgical antisepsis, an incision was made in the dorsal skin of the neck. Dorsal paravertebral muscles between C2 and T1 were incised and retracted and the posterior portion of cervical vertebrae was exposed. A right-sided laminectomy was performed either at C3 or C5 while preserving the facet joints. At either segment, the vertebral column was clamped one level above and one level below the desired contusion segment using an Infinite Horizons Impactor (Precision System and Instrumentation, LLC, Lexington, KY). Rats underwent a single contusion injury just lateral to midline with the 1.3 mm diameter impactor tip and a force of 100 kDynes. Laminectomy animals underwent a laminectomy at either C3 ($n = 7$) or C5 ($n = 3$), but no contusion was performed. In all cases, the dura mater was kept intact.

Muscle and skin layers were sutured using 3-0 polyglactin. Animals were allowed to recover from anesthesia and surgery before transfer to an individual cage. Animals were monitored on a daily basis, and measures were taken to avoid dehydration and to minimize pain.

Histological assessment of contusion injury and phrenic motoneuron number

Retrograde labeling of phrenic motoneurons was achieved by injecting cholera toxin subunit B (CTB; Invitrogen Corp., Carlsbad, CA) bilaterally into the pleural space through a transthoracic approach, as described previously.^{9,29,30,33} Each animal was injected

twice with 10 μ L on each side of the thorax 3 days before the terminal surgery. In two animals, intrapleural CTB injections were performed 3 days before contusion injury, and there was no apparent difference in the efficacy of retrograde labeling, consistent with previous results in the C2 hemisection model of cervical SCI.^{9,29,30,33}

At the terminal experiment (14 DPI), animals were anesthetized, euthanized by exsanguination, and the diaphragm muscle was resected *en bloc* and placed in 0.9% NaCl solution. After this, animals were administered heparin and transcardially perfused with 4% paraformaldehyde in phosphate-buffered saline (PBS). The spinal cord was resected from C2 to C7, post-fixed in 4% paraformaldehyde for 24 h, and placed in 25% sucrose in 0.1 M PBS (pH 7.4) at 4°C for 3 days. Transverse sections of the spinal cord were cut (70 μ m thick) and stored in 0.1 M PBS until use.

Spinal cord sections were systematically and randomly immunostained (every fourth section). Goat immunoglobulin G (IgG) anti-CTB (List Biological Laboratories Inc., Campbell, CA) and Cy3-conjugated donkey antigoat IgG (Jackson ImmunoResearch Laboratories Inc., Baltimore, PA) were used as primary and secondary antibodies, respectively. The specificity of these antibodies has been validated previously.³⁴

All stained spinal cord sections were visually inspected using a Nikon C1 confocal microscope equipped with a 561 nm solid state laser and appropriate filter combinations (Nikon Instruments Inc., Melville, NY). Sections showing histologic evidence of contusion lesion were imaged using a Nikon 20x oil-immersion lens (NA 0.75), and the entire spinal cord section was stitched using NIS-Elements software (Nikon Instruments). The area of contusion injury was determined manually for each section, and the overall volume of contusion comprising a cystic cavity was calculated from the sum of all injury volumes with truncated cone interpolation between consecutive sections.

In the present study, the segmental location of the injury site was verified using a rat spinal cord atlas.³⁵ Confocal images were obtained from all selected spinal cord sections using a 40x oil-immersion lens (NA 1.3) with a step size of 0.8 μ m. Retrogradely labeled phrenic motoneurons were individually identified from the image stack and counted manually. The total number of motoneurons for an animal was determined from all randomly sampled sections.

To examine the distribution of motoneurons along a rostrocaudal axis, motoneuron counts were binned in 10 equally distributed segments (beginning at C3 and ending at C6). In addition, phrenic motoneuron counts on the contralateral side of the spinal cord were used to normalize the total number of phrenic motoneurons per side for each animal, as done in previous studies.⁹

Assessment of diaphragm muscle denervation

At the terminal experiment, the diaphragm muscle was placed on a silicon rubber (Sylgard; DowCorning, Midland, MI)-coated Petri dish and stretched to $\sim 1.4\times$ resting length. Motor end plates were labeled by incubation in Alexa Fluor 488-conjugated α -bungarotoxin (0.1 μ g/mL; Invitrogen Corp.). Subsequently, diaphragm muscle samples were washed with 0.1M phosphate buffer, fixed with 4% paraformaldehyde, and stained for pre-synaptic structures using an antisynaptophysin antibody (1 mg/mL; SC-9116, Santa Cruz Biotechnology Inc., Santa Cruz, CA) and a Cy3-conjugated secondary antibody (donkey anti-rabbit IgG, Jackson Immuno Research Laboratories). The specificity of these toxins and antibodies has been validated previously.^{34,36–38}

Confocal microscopy was used for morphological analyses of neuromuscular junction (NMJ) denervation as in previous studies.^{36–40} Pixel dimensions (0.5 μ m $\times$ 0.5 μ m) were set to be above the optical resolution for a Nikon 40x water immersion lens (NA 0.8), avoiding oversampling. The Z-axis resolution was empirically determined from image stacks reconstructed in XZ, as reported previously.⁴¹ Step size was set to match the Z-axis resolution

(0.8 μm), and sequential images were obtained stepping in only one direction to avoid hysteresis in the stepper motor.

Individual NMJs were segregated based on their anatomical location into three zones corresponding to the ventral, middle, and dorsal portions of the diaphragm muscle (zones 1–3, respectively), based on the expected segmental innervation by the phrenic motoneuron pool.⁴² Confocal image stacks were obtained from superficial (within 50 μm of the surface) and *en face* NMJs to minimize fluorescence dispersion and to reduce the impact of anisotropic voxel dimensions on image reconstruction. Superficially located NMJs are not morphologically different from other NMJs located at great depths in the costal region of the rat diaphragm.^{36,37,39,43–48} As in previous studies,^{38,40} the volume of pre- and post-synaptic structures and the volume of apposition (as a percent of the post-synaptic volume) was obtained using a custom-designed algorithm in MetaMorph (Molecular Devices, LLC, Sunnyvale, CA).

Whole body plethysmography (WBP)

Barometric plethysmography (Buxco Inc, Wilmington, NC) was used to quantify ventilation in nonanesthetized animals before surgery and at 3, 7, and 14 DPI. The plethysmograph consisted of a Plexiglas chamber in which pressure was constantly monitored. The chamber's pressure, temperature, and humidity were calibrated before each experiment with pressurized gas mixtures flowing through the chambers at 10 L/min, thereby allowing control of inspired gas composition and preventing CO_2 buildup.

Rats were acclimatized for 20 min before recording a 10 min period of eupneic breathing. Rats were then exposed to hypoxic (10% inspired O_2) and hypercapnic (5% inspired CO_2) conditions for 5 min while continuously monitoring CO_2 concentration inside the chamber using a MicroCapStar capnograph (CWE Inc., Ardmore, PA). Plethysmography data for eupnea and hypoxia-hypercapnia were analyzed for 2 min periods after removing movement-related intervals, as in previous studies.³⁸ Ventilatory parameters analyzed were minute ventilation, tidal volume (V_T), and respiratory frequency.

Behavioral analysis

Animals were weighed on a daily basis up to 14 DPI. Open field Basso, Beattie and Bresnahan (BBB) locomotion scale⁴⁹ was used to assess impairment in hindlimb locomotion. The animals were placed in a transparent and empty cage for 5 min and allowed to freely move around; a score from 1 to 21 was given daily based on the characteristics of the locomotion.

Statistical analysis

All statistical evaluations were performed using JMP statistical software V 10.0 (SAS Institute Inc., Cary, NC). For measurements performed repeatedly on the same population (e.g., weight, WBP parameters, diaphragm EMG, and locomotion scales), comparisons were conducted using repeated measures analysis of variance (ANOVA) with time, experimental group, and, when appropriate, motor behavior (e.g., eupnea vs. hypoxia-hypercapnia) as grouping variables. Single outcome measures per animal (e.g., motoneuron counts, contusion force, and volume) were analyzed using a one-way ANOVA.

All experimental data are presented as mean $\pm$ standard deviation (SD), unless otherwise specified. Morphological data from individual NMJs were analyzed using a mixed linear model with animal as a random effect, experimental group and diaphragm zone as grouping variables. Least-squares means ($\pm$ standard error [SE]) are reported for morphological measurements, unless otherwise indicated. When appropriate, *post hoc* analyses were conducted using Tukey-Kramer honestly significant difference (HSD). A p value < 0.05 was considered statistically significant.

Results

Unilateral contusion of the cervical spinal cord

A total of 32 male rats were randomly assigned to one of three groups: laminectomy ($n = 12$), C3 contusion ($n = 10$), and C5 contusion ($n = 10$). At both cervical levels of contusion injuries, unilateral contusion was verified visually under the surgical microscope at the time of injury by the sidedness of the hematoma. Impacting force was not significantly different between the C3 and C5 contusion groups (119 ± 17 and 112 ± 9 kDynes, respectively; $p = 0.559$). There were also no differences in the displacement of the impactor tip within the spinal cord tissue ($1214 \pm 347 \mu\text{m}$ and $907 \pm 330 \mu\text{m}$ for the C3 and C5 contusion groups, respectively; $p = 0.121$).

There were no animal deaths during the procedure. All animals recovered uneventfully from surgery and anesthesia. No animals showed signs of blood in urine or stool after laminectomy or contusion surgery during the 14-day period of study.

Body weight was monitored daily before and after contusion. Overall, there was an effect of the experimental group and DPI on weight loss (two-way repeated measures ANOVA; $p < 0.001$ for group, DPI, and interaction). Animals in all groups lost 5–9% of body weight by 2 DPI with greater loss evident in the C3 contusion group compared with the laminectomy group ($p = 0.027$). After C3 contusion, maximal weight loss (9%) occurred by 2–4 DPI. Significant weight loss occurred by 3–4 DPI (13%) after C5 contusion ($p = 0.04$ vs. laminectomy). Animals regained their initial body weight by ~ 10 DPI such that body weight was $107 \pm 7\%$, $106 \pm 6\%$, and $103 \pm 11\%$ of initial weight at 14 DPI in the laminectomy, C3, and C5 contusion groups, respectively.

Contusion volume and phrenic motoneuron number

The level of histological injury was verified by comparison with standard spinal cord atlases, and two rats in the C5 contusion group were found not to have lesions at C5 but more caudally and were thus excluded from all analyses. Evidence of contusion injury was present unilaterally involving both gray matter in the dorsal and ventral horns (Fig. 1) and evidence of white matter injury restricted primarily to the dorsal and lateral funiculi. Anterior funiculi were consistently spared medially. Contusions at C3 or C5 had the level of maximum injury at C3/4 or C5/6, respectively. The extent of the

FIG. 1. Spinal cord histology after unilateral midcervical contusion injury. Representative 70 μm thick transverse spinal cord sections from an individual C3 and C5 contusion animal at 14 days post-injury. The level of maximum injury was the C3/C4 level for the C3 contusion and C5/C6 level for C5 contusion groups. In all cases, the pattern of cyst and scar formation compromised ventral and dorsal gray matter on the contused side only. A small notch on the left side was used to orient the sections (dorsal is on top). Bar, 1 mm.

lesion spanned 2–3 cervical levels. Cavity volumes were not significantly different between the C3 and C5 contusion groups (C3: $1.15 \pm 0.54 \mu\text{L}$ and C5: $1.25 \pm 0.75 \mu\text{L}$; $p=0.800$).

Retrogradely labeled phrenic motoneurons were counted in 17 animals using a systematic random sampling of spinal cord sections ($n=5$, 6, and 6 for the laminectomy, C3, and C5 contusion groups, respectively). There was a main effect of the experimental group on phrenic motoneuron counts ($p<0.001$) with no interaction with side ($p=0.301$). After laminectomy, phrenic motoneuron counts were 121 ± 33 and 116 ± 37 ipsi- and contralateral to surgery. Ipsilaterally, fewer phrenic motoneurons were evident after C3 contusion (45 ± 8 ; $p<0.05$) but did not reach statistical significance in the C5 contusion group (76 ± 36). No differences were evident in phrenic motoneuron counts contralateral to injury in either contusion group (79 ± 26 and 99 ± 60 in the C3 and C5 contusion groups, respectively). Phrenic motoneuron counts were also normalized to the contralateral side for each animal. On average, there were $39 \pm 13\%$ fewer motoneurons ipsilaterally following C3 contusion compared with laminectomy ($p=0.003$) and $13 \pm 21\%$ fewer motoneurons in the C5 group ($p=0.24$ vs. laminectomy).

To verify the sampling of phrenic motoneurons, a complete count was performed on one C3 contusion animal. A total of 172 and 258 phrenic motoneurons were found ipsi- and contralateral injury, representing 34% fewer phrenic motoneurons, consistent with the average reduction in motoneuron number in this experimental group.

The distribution of phrenic motoneurons across spinal cord levels was determined for each animal and side. For these analyses, the cervical spinal cord between C3 and C5 was partitioned into 10 levels. There was a fairly consistent distribution of motoneuron numbers across sides in the laminectomy group despite significant differences in cranial-caudal distribution (Fig. 2).

After C3 contusion, differences in phrenic motoneuron numbers were evident across sides in the most cranial levels (two-way ANOVA $F_{19,119} < 0.002$), with effect of segment ($p=0.044$) and side ($p<0.001$), but no interaction. Fewer phrenic motoneurons were evident ipsilaterally in the third level ($p=0.010$). Motoneuron count was not different at any level after C5 contusion. Similarly, there were no differences in the distribution of motoneurons contralateral to injury after C3 or C5 contusion.

Diaphragm muscle denervation

Individual diaphragm NMJs were visualized using fluorescence confocal microscopy and measured using a systematic random sampling from 23 animals ($n=8$, 3, 6, and 6 for the laminectomy, intact nonsurgical sham, C3, and C5 contusion groups, respectively). Confocal image stacks included ~ 30 images per NMJ, and overall ~ 45 NMJs were imaged per diaphragm. In all animals, excellent visualization of pre- and post-synaptic structures at the NMJ was evident using synaptophysin immunoreactivity and fluorescently conjugated α -bungarotoxin, respectively (Fig. 3).

For the purposes of evaluating diaphragm denervation, the diaphragm of each animal was divided into three zones (Fig. 3). The percentage of NMJs displaying evidence of denervation (e.g., partial overlap of pre- and post-synaptic structures) was not different between the laminectomy and intact nonsurgical sham groups (on average, $4.8 \pm 3.0\%$ for zone 1, $4.8 \pm 2.9\%$ for zone 2, $3.6 \pm 2.9\%$ for zone 3; $p \geq 0.357$ for group, zone, and interaction). Accordingly, these two groups were combined into a sham/lami-

FIG. 2. Distribution of phrenic motoneurons after unilateral C3 or C5 contusion injury. Phrenic motoneuron distribution across spinal cord levels C3 to C6 was similar in all three groups on the contralateral (intact) side. Differences in motoneuron counts across sides were only evident in the C3 contusion group (two-way analysis of variance, $p<0.002$). Error bars represent standard error.

nectomy group. Overall, $4.0 \pm 3.0\%$ of diaphragm NMJs showed signs of denervation in the sham/laminectomy group compared with $16.6 \pm 4.0\%$ of NMJs in the C3 and $7.1 \pm 3.6\%$ in the C5 contusion groups ($p=0.047$).

The difference between the C3 contusion and sham/laminectomy groups was statistically significant (Fig. 3), with a higher percentage of NMJs showing signs of denervation in zone 1 in the C3 contusion group compared with sham/laminectomy ($p<0.001$). Denervation after C5 contusion was not different from the sham/laminectomy group in any zone.

Morphometric analyses were conducted at individual diaphragm NMJs using three-dimensional reconstructions of pre- and post-synaptic structures to determine the volume of apposition (as a percent of the post-synaptic volume; Fig. 4). Differences in volume of apposition were evident in the linear mixed model with animal as a random effect ($p<0.001$), with significant differences evident across groups ($p=0.019$) and zones ($p=0.020$) with an interaction ($p<0.001$).

Overall, the volume of apposition was $45.6 \pm 3.0\%$ in the sham/laminectomy group, $30.9 \pm 4.1\%$ in the C3 contusion group, and $44.3 \pm 4.2\%$ in the C5 contusion group, with a leftward shift in the distribution in the C3 group compared with the other two experimental groups (Fig. 4). The volume of apposition in zone 1 was

FIG. 3. Diaphragm neuromuscular junction (NMJ) denervation after unilateral C3 or C5 contusion injury. Representative maximum intensity projections from an innervated (A) and denervated (B) NMJ labeled pre-synaptically with synaptophysin (red) and post-synaptically with Alexa Fluor 488-conjugated α -bungarotoxin. Bar, 10 μ m. (C) Intact diaphragm muscle showing midcostal and crural sections (crural at bottom). The midcostal diaphragm was divided into three zones in a ventral to dorsal direction in general agreement with predicted segmental innervation. (D) The percentage of NMJs showing denervation in the diaphragm overall as well as per zone in each group. NMJ denervation after C3 contusion was increased overall compared with sham/laminectomy (*, $p=0.047$). In the C3 contusion group, NMJ denervation was increased in zone 1 compared with zone 3 (†). In zone 1, NMJ denervation in the C3 contusion group was increased compared with the other two groups (‡). No difference in the percent of NMJ denervation was evident between the C5 contusion and sham/laminectomy groups for any zone. No difference across groups was evident in other zones. Error bars represent standard error.

lower compared with zone 3 (overall 40.2% vs. 44.1%, respectively), but only in the C3 contusion group was this difference statistically significant.

Diaphragm EMG activity

Diaphragm EMG was recorded chronically to determine rhythmic EMG activity after laminectomy ($n=8$), C3 contusion ($n=6$), or C5 contusion ($n=7$). For eupnea and sigh measurements in each animal, diaphragm RMS EMG amplitude was normalized to the sigh value at 0 DPI for the same side. As expected, normalized diaphragm RMS EMG amplitude in the laminectomy group was stable over time. Normalized RMS EMG amplitude during eupnea was $28 \pm 10\%$ compared with $31 \pm 17\%$ at 0 and 14 DPI, respectively ($p>0.548$). Normalized RMS EMG amplitude during sigh was $121 \pm 10\%$ at 14 DPI.

No differences in normalized RMS EMG amplitude were evident over time in the C3 contusion ($p>0.671$) or C5 contusion groups ($p>0.169$). No differences in normalized RMS EMG amplitude were evident across groups at 0 DPI (laminectomy: $28 \pm 3\%$; C3 contusion: $29 \pm 6\%$; C5 contusion: $23 \pm 6\%$; $p>0.160$ for both ipsilateral and contralateral comparisons). Contralateral eupneic RMS EMG amplitude increased transiently at 1 DPI compared with other groups ($41 \pm 11\%$ for the C3 contusion vs. $27 \pm 8\%$ for laminectomy and $28 \pm 7\%$ for C5 contusion groups—Fig. 5; one-way ANOVA; $F_{2,18}=4.60$; $p=0.026$).

No differences across groups in normalized eupneic RMS EMG amplitude were evident at any other time point ($p>0.173$ for both ipsilateral and contralateral comparisons). In the C3 and C5 contusion groups, diaphragm RMS EMG amplitude during sighs did

not change over time up to 14 DPI ($p>0.438$ for both ipsilateral and contralateral comparisons).

Whole body plathysmography

In awake, unrestrained rats from each group, WBP was used to analyze V_T and minute ventilation during eupnea and hypoxia-hypercapnia at 0, 3, 7, and 14 DPI ($n=10$, 9, and 5 for the laminectomy, C3, and C5 contusion groups, respectively). No significant differences in eupneic ventilation were evident across groups over time (repeated-measures ANOVA; $p=0.296$). At 0 DPI, eupneic minute ventilation was 66 ± 16 mL/min/100 g for all the groups, with no difference across groups. At 14 DPI, eupneic minute ventilation was 77 ± 11 mL/min/100 g in the laminectomy, 91 ± 30 mL/min/100 g in the C3 contusion and 125 ± 36 mL/min/100 g in the C5 contusion groups; however, the difference between groups was not significant ($p=0.083$).

Eupneic V_T also did not change over time or across groups (Fig. 6; $p=0.705$). At 0 DPI, V_T was 0.71 ± 0.15 mL/ breath/100 g on average for all the groups, with no difference across groups. At 14 DPI, V_T was 0.79 ± 0.15 mL/ breath/100 g on average for all the groups.

All of the groups showed the same response during all days to the hypoxia-hypercapnia stimuli, increasing minute ventilation values two- to three-fold and almost doubling V_T values (two-way repeated measures ANOVA; $p<0.001$ vs. eupnea). On average, minute ventilation during hypoxia-hypercapnia at 0 DPI for all groups was 182 ± 53 mL/min/100 g and V_T was 1.28 ± 0.27 mL/ breath/100 g.

There was an effect of time and no effect of group on minute ventilation during hypoxia-hypercapnia ($p=0.039$ for time and

FIG. 4. Morphometric assessment of NMJ denervation after unilateral C3 or C5 contusion injury. **(A)** Binarized images showing three-dimensional rendering of pre- and post-synaptic volumes for representative innervated (top) and denervated (bottom) NMJs in Figure 3, with varying depth represented by grayscale intensity (scale bar at right). Images represent the volume of apposition between pre- and post-synaptic structures, with depth shown by scale at right. Bar, 10 μ m. **(B)** Cumulative probability plot for volume of apposition (as a percent of post-synaptic volume). Reduced pre- vs. post-synaptic apposition is evident for the C3 contusion group compared with other groups (left shifted distribution). **(C)** Volume of apposition across zones and groups. After C3 contusion, NMJs in zone 1 had lower volume of apposition than in zone 3 (*, $p < 0.001$). No other differences were evident across groups or zones. Error bars represent standard error.

$p = 0.367$ for group). Average minute ventilation during hypoxia-hypercapnia at 14 DPI was 233 ± 64 mL/min/100 g. V_T during hypoxia-hypercapnia did not change over time, and there was no difference across groups (Fig. 6; $p = 0.654$ for time and $p = 0.121$ for group).

Behavioral analysis

BBB locomotion scale (21 point scale) was measured daily on 26 animals ($n = 11$, 9, and 6 for the laminectomy, C3, and C5 contusion groups, respectively). Both C3 and C5 contusion groups showed a decrease at 1 DPI with an average score of 8 ± 1 (Fig. 7). No significant difference was found between the two contusion groups, and both had a decreased score compared with the laminectomy group from 1–9 DPI ($p < 0.001$). By 14 DPI, C3 and C5 contusion animals scored on average 19 ± 3 . The laminectomy group showed a nonsignificant decrease of 1 point by 1 DPI, and all the animals in this group had a maximal score by 3 DPI.

Discussion

The present study compared two different contusion injuries of the midcervical spinal cord and their effects on ipsilateral rhythmic diaphragm activity. We hypothesized that a unilateral contusion injury at C3 versus C5 would differentially impact phrenic (diaphragm EMG) activity reflecting more prominent disruption of ipsilateral descending excitatory drive to more caudal segments of the phrenic motor pool with more cranial injuries, with minimal evidence of impaired ventilatory function. The unilateral contusion injury performed in this study resulted in evidence of cystic cavity formation and locomotion impairments that were similar between the C3 and C5 contusion groups.

Despite evidence of spinal cord injury, phrenic motoneuron loss, and diaphragm denervation, there was minimal functional compromise of ventilation after either C3 or C5 contusion. Indeed, tidal volume and minute ventilation as assessed by WBP were unaffected by either contusion level, and diaphragm RMS EMG amplitude showed only transient contralateral compensation acutely

FIG. 5. Chronic diaphragm electromyographic (EMG) activity before and after unilateral C3 or C5 contusion injury. (A) Representative raw diaphragm EMG recordings and root mean square (RMS) EMG tracings from a C3 contusion animal monitored during sigh and eupnea at 0 and 1 days post-injury (DPI). (B) Diaphragm RMS EMG amplitude at 0, 1, 3, 7, and 14 DPI normalized to RMS EMG amplitude during sigh at 0 DPI. At 1 DPI, C3 contusion resulted in increased contralateral RMS EMG amplitude compared with laminectomy or C5 contusion groups (*, $p=0.026$). No changes in RMS EMG amplitude were observed over time on the ipsilateral side in any group.

after C3 contusion. These results are consistent with a large reserve capacity for force generation by the diaphragm muscle^{19–22} and the minimal impairment in ventilation immediately after unilateral phrenic denervation.²³

Contusion injury and phrenic motoneuron loss

Unilateral midcervical contusion injuries at C3 and C5 exhibited a similar extent of spinal cord damage; at 14 DPI, lesioned tissue comprised the ventral and dorsal gray matter and dorsolateral funiculi of white matter with no compromise of the contralateral side. The volume of the post-traumatic cystic cavity was $\sim 1.2 \mu\text{L}$ in both the C3 and C5 contusion groups. After a 150 kDynes unilateral contusion at C3/4, lesion volume was reported as $<0.1 \mu\text{L}$ at 21 DPI.⁵⁰ After a 400 kDynes unilateral contusion at C4, lesion volume was reported in a range of 3 to 13 μL at 10–14 DPI.^{15,17,51,52} Lesion volume after double contusion (~ 400 kDynes) at C3 and C4 was $\sim 4 \mu\text{L}$ primarily reflecting cranial-caudal lesion extent at 6 weeks post-injury.¹⁵

Other studies have not consistently reported similar measures of lesion volume.^{12–14} Regardless, there is substantial variability in

FIG. 6. Tidal volume (V_T) measurements using whole body plethysmography at 0, 3, 7, and 14 days post-injury. No changes in V_T were observed over time after unilateral C3 or C5 contusion injury compared with the laminectomy group. All three groups showed a two-fold increase from eupnea to hypoxia-hypercapnia on all days evaluated. Data shown as mean $\pm$ standard error.

FIG. 7. Locomotion analysis after unilateral C3 or C5 contusion injury, as measured by the 21-point Basso, Beattie and Bresnahan (BBB) scale. Animals were measured daily from 0 to 14 days post-injury (DPI). Both C3 and C5 contusion groups had lower scores compared with the laminectomy group from 1 to 9 DPI ($p<0.001$). There was no difference in BBB scores between the two contused groups at any time post-injury. Data shown as mean $\pm$ standard error.

lesion volume. The current study used a smaller impactor tip size (1.3 mm diameter) compared with other unilateral C3/4 contusion studies (1.5 mm), and the 100 kDynes impact force was less than other studies using 150 kDynes^{18,50} or 395 kDynes.^{15,17,51,52} All these studies, however, including the present study, inflicted damage to both gray and white matter. The extent of gray matter injury was associated with evidence of motoneuron loss. Although white matter injury was evident in all animals in the present study, the extent of disruption of descending bulbospinal pathways after either C3 or C5 contusion injury cannot be unambiguously assessed using histopathological measurements.

The present study focused on phrenic motoneuron loss using a previously validated retrograde labeling technique⁹ and a stereological systematic random sampling approach.^{53,54} In rats, the phrenic motoneuron pool is located between the C3 to C5 segments.^{8,9} Previous studies quantifying motoneuron loss after moderate to severe midcervical contusion suggested a fairly homogeneous distribution of the phrenic motoneurons across these spinal segments.^{15–17}

The present study, however, demonstrates that phrenic motoneurons are not homogeneously distributed between C3 to C5, with most motoneurons located between C3 and C4 and some motoneurons present as caudally as C6. A significant loss in the total number of phrenic motoneurons was evident after a C3 contusion, with lost motoneurons segmentally in the phrenic nucleus between the C3 and C4 segments.

In our injury model, the most cranial phrenic motoneurons were spared by C3 contusion, reflecting the surgical approach via a C3 laminectomy. At 14 DPI after the 100 kDynes unilateral mid-cervical contusion, there was $\sim 40\%$ reduction in the numbers of phrenic motoneurons in the C3 contusion group. Of note, 14 and 42 DPI after a single 400 kDynes unilateral contusion at C4 or double contusion at C3 and C4, there was a similar reduction (40–50%) in phrenic motoneuron numbers.¹⁵ After C5 contusion, there was no difference in motoneuron numbers at any spinal cord segment by 14 DPI despite $\sim 15\%$ fewer phrenic motoneurons.

Greater than 50% of the phrenic motoneuron pool was reported to be located within the region of injury after bilateral midcervical contusion,¹² although motoneuron loss was not specifically evaluated in that study. At this point, it is not clear whether motoneuron

loss occurs early (within 1 DPI)¹⁷ after less severe injury models such as that used in the present study.

Evidence of diaphragm muscle denervation

In the current study, C3 contusion resulted in greater denervation (17%) than the laminectomy (4%) or C5 contusion group (7%). Denervation across diaphragm muscle zones was consistent with the branching innervation⁴²; greater denervation was evident ventrally after C3 contusion and dorsally after C5 contusion. The results are consistent with previous studies that have assessed diaphragm muscle denervation after spinal cord contusion.^{15–17,51}

Of note, the single 400 kDynes unilateral contusion at C4 resulted in ~40% denervation, primarily in ventral regions but also some dorsally, consistent with phrenic motoneuron loss. After a double contusion at C3 and C4, however, NMJ denervation was reported across the entire hemidiaphragm, with 100% denervation in ventral regions, even though phrenic motoneuron loss was not significantly greater at 14 DPI.

Future studies should help elucidate the mechanisms of diaphragm denervation as it relates to motoneuron injury associated with retraction of axon terminals and denervation but not frank loss of motoneurons. In this regard, detailed morphological analyses such as those conducted in the present study might be particularly more informative (e.g., volume of apposition) rather than the more commonly employed qualitative assessments.

Minimal functional impairment after unilateral midcervical contusion injury

Despite evidence of motoneuron loss and diaphragm denervation, there is minimal functional impairment after a unilateral midcervical contusion. The laminectomy group displayed small, expected levels of variation in the EMG signal amplitude,²⁷ consistent with changes in chronic EMG recordings because of animal growth, electrode movement, or scarring of the tissue, among other reasons.

No change was seen in the C3 contusion group for either normal breathing or sigh EMG amplitude on the contused side; however, an immediate but transient compensatory increase in EMG amplitude was observed on the contralateral side. Generally similar increases in contralateral EMG activity were evident after ipsilateral hemidiaphragm paralysis induced by unilateral C2 spinal hemisection^{28,39,55} or acute denervation.²³ After C5 contusion the EMG amplitude did not change on either side for any behavior. These results are in agreement with a previous study demonstrating no change in EMG amplitude at 14 DPI after unilateral C4 contusion.¹⁵

A single, common site for EMG electrode placement was used in the present study (midcostal), and the segmental innervation of the diaphragm muscle (C3 ventrally vs. C5 dorsally) should be considered. A recent study analyzed EMG activity separately at dorsal, medial, or ventral regions of the diaphragm muscle and showed that integrated diaphragm EMG amplitude varied across regions at 10 DPI after unilateral C4 contusion.⁵¹ In their study, Li and associates⁵¹ did not conduct chronic EMG recordings and thus did not examine pre-contusion EMG activity, and a control or sham injury was not included to establish that these differences in EMG amplitude indeed reflect the injury condition.

Importantly, longitudinal analysis of diaphragm EMG activity using chronically implanted electrodes is necessary to permit quantitative assessment of diaphragm muscle function over time, and these measures can be enhanced by comparisons across motor behaviors and normalization to a maximum.²⁷

Previous studies have reported substantial functional impairment after cervical contusion injuries primarily using various measurements of phrenic nerve activity.^{12–15,17} After unilateral C4 contusion, phrenic nerve compound muscle action potential amplitude decreased immediately at 1 DPI and remained decreased through 14 DPI.^{15,17} At 5 weeks after unilateral C2 contusion, el-Bohy and colleagues¹⁴ found a decrease in phrenic nerve inspiratory output during asphyxia on the injured side.

Golder and coworkers¹³ found a decrease in phrenic inspiratory output on the injured side during hypoxia-hypercapnia at 14 days after unilateral C4–C5 contusion. Recordings were only possible, however, at the terminal experiment, thus precluding analyses within individual animals. In this sense, previous results are fundamentally different from the current study, which chronically measured diaphragm EMG amplitude over time to assess changes that occur in the same measurement before and after injury.

The diaphragm muscle is active across a range of ventilatory and nonventilatory behaviors with varying levels of force generation. During ventilation in normoxic and hypoxic or hypercarbic conditions, recruitment of only a relatively small fraction of the phrenic motor unit pool is necessary to generate the forces associated with these behaviors.^{21,22,56,57}

Indeed, a recent study found that unilateral diaphragm muscle denervation does not affect diaphragm force generation (as assessed by transdiaphragmatic pressure [Pdi]) during eupnea and hypoxia-hypercapnia, whereas Pdi generated during higher force, non-ventilatory behaviors (e.g., sigh, tracheal occlusion) was significantly reduced.²³ Given that the forces generated during sighs (~65% of the maximal Pdi elicited by bilateral phrenic nerve stimulation in rats) require recruitment of ~85% of the motor unit pool,⁵⁶ we expected that midcervical contusions would decrease EMG amplitude. It is possible that ongoing reinnervation of muscle fibers (since evidence of denervation was more modest than the evidence of motoneuron loss) may contribute to preservation of diaphragm muscle force.

The lack of evident functional impairment using EMG during ventilatory behaviors suggests that assessment of higher force behaviors (i.e., airway occlusion or sneezing) and normalization to maximum force may be needed to reveal a change in EMG amplitude after partial injuries to the phrenic motoneuron pool as seen after spinal cord contusion or other models of toxic motoneuron death.⁵⁸ Overall, these results are consistent with a large reserve capacity for ventilatory functions of the diaphragm muscle.

There was no change in minute ventilation or V_T after C3 or C5 contusion during either eupneic or hypoxic-hypercapnic breathing. Previous studies reported changes in WBP after both unilateral^{17,59} and bilateral^{12,13} midcervical contusion. At 14 DPI, V_T during eupnea but not during hypercapnia (7% CO_2) was negatively correlated ($r^2=0.85$) with the percent of injury area in the spinal cord.¹³ Minute ventilation was reduced, however, in the bilaterally contused animals only at 2 DPI, not 14 DPI, and during exposure to hypercapnia but not in room air.

Choi and colleagues⁵⁹ found reductions in V_T and minute ventilation in room air through 14 DPI and in hypercapnic challenge (7% CO_2) through 4 weeks after unilateral C5 contusion. Nicaise and associates¹⁷ found a decrease in V_T , peak expiratory flow, and expiratory time and an increase in frequency immediately after unilateral injury; however, this change was transient and returned to pre-injury values at 4 DPI. Also consistent with our findings, Lane and coworkers¹² did not find a change in any WBP parameter 1 to 10 weeks after a midline C3/4 contusion.

After acute unilateral denervation of the diaphragm muscle, there is a decrease in V_T and an increase in frequency,²³ but all of

these changes are relatively small (less than 20%). Importantly, ventilatory assessments using WBP are limited by the contribution of unilateral impairments in diaphragm muscle force generation being masked by contralateral diaphragm activity as well as the possible activation of respiratory accessory muscles.

Regardless, the lack of functional impairment of ventilatory behaviors after contusion injury is significant in light of the substantial loss of phrenic motoneurons (~40%). Ventilation after contusion injuries likely depends primarily on the number of phrenic motoneurons that are lost, more than on the extent of spared bulbospinal excitatory drive (both ipsilateral and contralateral) to surviving motoneurons. Thus, even if bulbospinal input was totally spared in the 100 kDynes unilateral C3 or C5 contusion (unlikely based on the histomorphological measurements conducted) as is after acute unilateral phrenic denervation,²³ the finding that ventilation was unimpaired during eupnea and hypoxia-hypercapnia highlights the large reserve capacity for force generation of the diaphragm muscle and the importance of assessing higher force behaviors (e.g., airway occlusion or sneezing).

Body weight variation and locomotion impairment after contusion

Consistent with injury to mid-cervical spinal cord segments, animals displayed impaired changes in body weight and locomotion. Although both C3 and C5 contusion groups showed greater weight loss compared to the laminectomy group, animals regained their initial body weight by ~10 DPI. No difference in overall weight loss was found between the two contusion groups, but the C5 contusion group showed weight loss for a longer time than the C3 contusion group (2 days longer). In general agreement, after a 400 kDynes unilateral contusion at C4, animals regained their initial body weight by ~14 DPI.¹⁵ Locomotion assessed using the BBB score was also affected after contusion; both C3 and C5 contusion groups had a decreased BBB score compared to laminectomy group from 1–9 DPI. In previous studies, ipsilateral forelimb grip strength was reduced through 14 DPI after a 400 kDynes C4 contusion,¹⁵ but near intact forelimb function was reported within 7–14 DPI of either 150 or 250 kDynes C3/4 midline contusion.¹²

Research implications

Diaphragm motor behaviors including ventilation during eupnea and hypoxia-hypercapnia require recruitment of only a fraction of the total phrenic motor unit pool (~45% in rats⁵⁶). In agreement, increased contralateral diaphragm activity at 1 DPI is consistent with impaired force development ipsilateral to C3 contusion injury. However, there was no evidence of increased contralateral activity during sighs, which require a substantially greater fraction of the motor unit pool (~85% in rats⁵⁶). Of note, there also was no change in contralateral diaphragm RMS EMG activity after complete unilateral hemidiaphragm paralysis induced by acute phrenic denervation.²³ Thus, it seems that measurements during high force behaviors involving activation of the entire motor unit pool (e.g., sneezing) will be necessary to evaluate functional compromise of the diaphragm muscle. Clinically, significant changes in functional outcomes are evident with a difference in two mid-cervical spinal cord segments.^{10,11} However, such clinical outcomes do not consistently account for differences in the ability to sustain independent ventilation, and emphasize upper extremity function as it relates to independence in self-care, wheel chair management and transfers. Based on the results of the present contusion study,

substantial resilience in respiratory function is evident and does not reflect changes across cervical spinal cord levels.

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Author Disclosure Statement

No competing financial interests exist.

References

- Hartkopp, A., Bronnum-Hansen, H., Seidenschner, A.M., and Biering-Sorensen, F. (1997). Survival and cause of death after traumatic spinal cord injury. A long-term epidemiological survey from Denmark. *Spinal Cord* 35, 76–85.
- Brown, R., DiMarco, A.F., Hoit, J.D. and Garshick, E. (2006). Respiratory dysfunction and management in spinal cord injury. *Respir. Care* 51, 853–870.
- Lidal, I.B., Snekkvik, H., Aamodt, G., Hjeltne, N., Biering-Sorensen, F., and Stanghelle, J.K. (2007). Mortality after spinal cord injury in Norway. *J. Rehabil. Med.* 39, 145–151.
- Dobbins, E.G., and Feldman, J.L. (1994). Brainstem network controlling descending drive to phrenic motoneurons in rat. *J. Comp. Neurol.* 347, 64–86.
- Feldman, J.L., Loewy, A.D., and Speck, D.F. (1985). Projections from the ventral respiratory group to phrenic and intercostal motoneurons in cat: an autoradiographic study. *J. Neurosci.* 5, 1993–2000.
- Ellenberger, H.H., and Feldman, J.L. (1988). Monosynaptic transmission of respiratory drive to phrenic motoneurons from brainstem bulbospinal neurons in rats. *J. Comp. Neurol.* 269, 47–57.
- Keswani, N.H., and Hollinshead, W.H. (1955). The phrenic nucleus. III. Organization of the phrenic nucleus in the spinal cord of the cat and man. *Proc. Staff Meet. Mayo Clin.* 30, 566–577.
- Goshgarian, H.G., and Rafols, J.A. (1981). The phrenic nucleus of the albino rat: a correlative HRP and Golgi study. *J. Comp. Neurol.* 201, 441–456.
- Mantilla, C.B., Zhan, W.Z., and Sieck, G.C. (2009). Retrograde labeling of phrenic motoneurons by intrapleural injection. *J. Neurosci. Methods* 182, 244–249.
- Kramer, J.L., Lammertse, D.P., Schubert, M., Curt, A., and Steeves, J.D. (2012). Relationship between motor recovery and independence after sensorimotor-complete cervical spinal cord injury. *Neurorehabil. Neural Repair.* 26, 1064–1071.
- van Hedel, H.J., and Curt, A. (2006). Fighting for each segment: estimating the clinical value of cervical and thoracic segments in SCI. *J. Neurotrauma* 23, 1621–1631.
- Lane, M.A., Lee, K.Z., Salazar, K., O'Steen, B.E., Bloom, D.C., Fuller, D.D., and Reier, P.J. (2012). Respiratory function following bilateral mid-cervical contusion injury in the adult rat. *Exp. Neurol.* 235, 197–210.
- Golder, F.J., Fuller, D.D., Lovett-Barr, M.R., Vinit, S., Resnick, D.K., and Mitchell, G.S. (2011). Breathing patterns after mid-cervical spinal contusion in rats. *Exp. Neurol.* 231, 97–103.
- el-Bohy, A.A., Schrimsher, G.W., Reier, P.J., and Goshgarian, H.G. (1998). Quantitative assessment of respiratory function following contusion injury of the cervical spinal cord. *Exp. Neurol.* 150, 143–152.
- Nicaise, C., Hala, T.J., Frank, D.M., Parker, J.L., Authalet, M., Leroy, K., Brion, J.P., Wright, M.C., and Lepore, A.C. (2012). Phrenic motor neuron degeneration compromises phrenic axonal circuitry and diaphragm activity in a unilateral cervical contusion model of spinal cord injury. *Exp. Neurol.* 235, 539–552.
- Nicaise, C., Putatunda, R., Hala, T.J., Regan, K.A., Frank, D.M., Brion, J.P., Leroy, K., Pochet, R., Wright, M.C., and Lepore, A.C. (2012). Degeneration of phrenic motor neurons induces long-term diaphragm deficits following mid-cervical spinal contusion in mice. *J. Neurotrauma* 29, 2748–2760.
- Nicaise, C., Frank, D.M., Hala, T.J., Authalet, M., Pochet, R., Adriaens, D., Brion, J.P., Wright, M.C., and Lepore, A.C. (2013). Early phrenic motor neuron loss and transient respiratory abnormalities after unilateral cervical spinal cord contusion. *J. Neurotrauma* 30, 1092–1099.

18. Awad, B.I., Warren, P.M., Steinmetz, M.P., and Alilain, W.J. (2013). The role of the crossed phrenic pathway after cervical contusion injury and a new model to evaluate therapeutic interventions. *Exp. Neurol.* 248, 398–405.
19. Mantilla, C.B., Seven, Y.B., and Sieck, G.C. (2014). Convergence of pattern generator outputs on a common mechanism of diaphragm motor unit recruitment. *Prog. Brain Res.* 209, 309–329.
20. Mantilla, C.B., and Sieck, G.C. (2011). Phrenic motor unit recruitment during ventilatory and non-ventilatory behaviors. *Respir. Physiol. Neurobiol.* 179, 57–63.
21. Sieck, G.C. (1994). Physiological effects of diaphragm muscle denervation and disuse. *Clin. Chest Med.* 15, 641–659.
22. Sieck, G.C., and Fournier, M. (1989). Diaphragm motor unit recruitment during ventilatory and nonventilatory behaviors. *J. Appl. Physiol.* 66, 2539–2545.
23. Gill, L.C., Mantilla, C.B., and Sieck, G.C. (2015). Impact of unilateral denervation on transdiaphragmatic pressure. *Respir. Physiol. Neurobiol.* 210, 14–21.
24. Dow, D.E., Mantilla, C.B., Zhan, W.Z., and Sieck, G.C. (2006). EMG-based detection of inspiration in the rat diaphragm muscle. *Conf. Proc. IEEE Eng. Med. Biol. Soc.* 1, 1204–1207.
25. Dow, D.E., Zhan, W.Z., Sieck, G.C., and Mantilla, C.B. (2009). Correlation of respiratory activity of contralateral diaphragm muscles for evaluation of recovery following hemiparesis. *Conf. Proc. IEEE Eng. Med. Biol. Soc.* 2009, 404–407.
26. Trelease, R.B., Sieck, G.C., and Harper, R.M. (1982). A new technique for acute and chronic recording of crural diaphragm EMG in cats. *Electroencephalogr. Clin. Neurophysiol.* 53, 459–462.
27. Mantilla, C.B., Seven, Y.B., Hurtado-Palomino, J.N., Zhan, W.Z., and Sieck, G.C. (2011). Chronic assessment of diaphragm muscle EMG activity across motor behaviors. *Respir. Physiol. Neurobiol.* 177, 176–182.
28. Mantilla, C.B., Greising, S.M., Zhan, W.Z., Seven, Y.B. and Sieck, G.C. (2013). Prolonged C2 spinal hemisection-induced inactivity reduces diaphragm muscle specific force with modest, selective atrophy of type IIX and/or IIB fibers. *J. Appl. Physiol.* 114, 380–386.
29. Gransee, H.M., Zhan, W.Z., Sieck, G.C., and Mantilla, C.B. (2015). Localized delivery of brain-derived neurotrophic factor-expressing mesenchymal stem cells enhances functional recovery following cervical spinal cord injury. *J. Neurotrauma* 32, 185–193.
30. Mantilla, C.B., Gransee, H.M., Zhan, W.Z., and Sieck, G.C. (2013). Motoneuron BDNF/TrkB signaling enhances functional recovery after cervical spinal cord injury. *Exp. Neurol.* 247, 101–109.
31. Seven, Y.B., Mantilla, C.B., Zhan, W.Z., and Sieck, G.C. (2013). Non-stationarity and power spectral shifts in EMG activity reflect motor unit recruitment in rat diaphragm muscle. *Respir. Physiol. Neurobiol.* 185, 400–409.
32. Seven, Y.B., Mantilla, C.B., and Sieck, G.C. (2014). Recruitment of rat diaphragm motor units across motor behaviors with different levels of diaphragm activation. *J. Appl. Physiol.* 117, 1308–1316.
33. Gransee, H.M., Zhan, W.Z., Sieck, G.C., and Mantilla, C.B. (2013). Targeted delivery of TrkB receptor to phrenic motoneurons enhances functional recovery of rhythmic phrenic activity after cervical spinal hemisection. *PLoS One* 8, e64755.
34. Issa, A.N., Zhan, W.Z., Sieck, G., and Mantilla, C.B. (2010). Neuregulin-1 at synapses on phrenic motoneurons. *J. Comp. Neurol.* 518, 4213–4225.
35. Sengul, G., Watson, C., Tanaka, I., and Paxinos, G. 2012. *Atlas of the Spinal Cord: Mouse, Rat, Rhesus, Marmoset, and Human*. Academic Press: San Diego.
36. Mantilla, C.B., Rowley, K.L., Fahim, M.A., Zhan, W.Z., and Sieck, G.C. (2004). Synaptic vesicle cycling at type-identified diaphragm neuromuscular junctions. *Muscle Nerve* 30, 774–783.
37. Mantilla, C.B., Rowley, K.L., Zhan, W.Z., Fahim, M.A., and Sieck, G.C. (2007). Synaptic vesicle pools at diaphragm neuromuscular junctions vary with motoneuron soma, not axon terminal, inactivity. *Neuroscience* 146, 178–189.
38. Sieck, D.C., Zhan, W.Z., Fang, Y.H., Ermilov, L.G., Sieck, G.C., and Mantilla, C.B. (2012). Structure-activity relationships in rodent diaphragm muscle fibers vs. neuromuscular junctions. *Respir. Physiol. Neurobiol.* 180, 88–96.
39. Prakash, Y.S., Miyata, H., Zhan, W.Z., and Sieck, G.C. (1999). Inactivity-induced remodeling of neuromuscular junctions in rat diaphragmatic muscle. *Muscle Nerve* 22, 307–319.
40. Mantilla, C.B., Stowe, J.M., Sieck, D.C., Ermilov, L.G., Greising, S.M., Zhang, C., Shokat, K.M., and Sieck, G.C. (2014). TrkB kinase activity maintains synaptic function and structural integrity at adult neuromuscular junctions. *J. Appl. Physiol.* 117, 910–920.
41. Sieck, G.C., Mantilla, C.B., and Prakash, Y.S. (1999). Volume measurements in confocal microscopy. *Methods Enzymol.* 307, 296–315.
42. Laskowski, M.B., and Sanes, J.R. (1987). Topographic mapping of motor pools onto skeletal muscles. *J. Neurosci.* 7, 252–260.
43. Prakash, Y.S., Gosselin, L.E., Zhan, W.Z., and Sieck, G.C. (1996). Alterations of diaphragm neuromuscular junctions with hypothyroidism. *J. Appl. Physiol.* 81, 1240–1248.
44. Prakash, Y.S., Miller, S.M., Huang, M., and Sieck, G.C. (1996). Morphology of diaphragm neuromuscular junctions on different fibre types. *J. Neurocytol.* 25, 88–100.
45. Prakash, Y.S., Zhan, W.Z., Miyata, H., and Sieck, G.C. (1995). Adaptations of diaphragm neuromuscular junction following inactivity. *Acta Anat.* 154, 147–161.
46. Prakash, Y.S., and Sieck, G.C. (1998). Age-related remodeling of neuromuscular junctions on type-identified diaphragm fibers. *Muscle Nerve* 21, 887–895.
47. Rowley, K.L., Mantilla, C.B., Ermilov, L.G., and Sieck, G.C. (2007). Synaptic vesicle distribution and release at rat diaphragm neuromuscular junctions. *J. Neurophysiol.* 98, 478–487.
48. Sieck, G.C., and Prakash, Y.S. (1997). Morphological adaptations of neuromuscular junctions depend on fiber type. *Can. J. Appl. Physiol.* 22, 197–230.
49. Basso, D.M., Beattie, M.S., and Bresnahan, J.C. (1995). A sensitive and reliable locomotor rating scale for open field testing in rats. *J. Neurotrauma* 12, 1–21.
50. Hoy, K.C. Jr., and Alilain, W.J. (2015). Acute theophylline exposure modulates breathing activity through a cervical contusion. *Exp. Neurol.* 271, 72–76.
51. Li, K., Javed, E., Hala, T.J., Sannie, D., Regan, K.A., Maragakis, N.J., Wright, M.C., Poulsen, D.J., and Lepore, A.C. (2015). Transplantation of glial progenitors that overexpress glutamate transporter GLT1 preserves diaphragm function following cervical SCI. *Mol. Ther.* 23, 533–548.
52. Li, K., Nicaise, C., Sannie, D., Hala, T.J., Javed, E., Parker, J.L., Putatunda, R., Regan, K.A., Suain, V., Brion, J.P., Rhoderick, F., Wright, M.C., Poulsen, D.J., and Lepore, A.C. (2014). Overexpression of the astrocyte glutamate transporter GLT1 exacerbates phrenic motor neuron degeneration, diaphragm compromise, and forelimb motor dysfunction following cervical contusion spinal cord injury. *J. Neurosci.* 34, 7622–7638.
53. Prakash, Y.S., Smithson, K.G., and Sieck, G.C. (1993). Measurements of motoneuron somal volumes using laser confocal microscopy: comparisons with shape-based stereological estimations. *Neuroimage* 1, 95–107.
54. Prakash, Y.S., Smithson, K.G., and Sieck, G.C. (1994). Application of the Cavalieri principle in volume estimation using laser confocal microscopy. *Neuroimage* 1, 325–333.
55. Miyata, H., Zhan, W.Z., Prakash, Y.S., and Sieck, G.C. (1995). Myoneural interactions affect diaphragm muscle adaptations to inactivity. *J. Appl. Physiol.* 79, 1640–1649.
56. Mantilla, C.B., Seven, Y.B., Zhan, W.Z., and Sieck, G.C. (2010). Diaphragm motor unit recruitment in rats. *Respir. Physiol. Neurobiol.* 173, 101–106.
57. Greising, S.M., Sieck, D.C., Sieck, G.C., and Mantilla, C.B. (2013). Novel method for transdiaphragmatic pressure measurements in mice. *Respir. Physiol. Neurobiol.* 188, 56–59.
58. Nichols, N.L., Vinit, S., Bauernschmidt, L., and Mitchell, G.S. (2015). Respiratory function after selective respiratory motor neuron death from intrapleural CTB-saporin injections. *Exp. Neurol.* 267, 18–29.
59. Choi, H., Liao, W.L., Newton, K.M., Onario, R.C., King, A.M., Desilets, F.C., Woodard, E.J., Eichler, M.E., Frontera, W.R., Sabharwal, S., and Teng, Y.D. (2005). Respiratory abnormalities resulting from midcervical spinal cord injury and their reversal by serotonin 1A agonists in conscious rats. *J. Neurosci.* 25, 4550–4559.

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