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Respiratory function following bilateral mid-cervical contusion injury in the adult rat

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Abstract

The consequences of spinal cord injury (SCI) are often viewed as the result of white matter damage. However, injuries occurring at any spinal level, especially in cervical and lumbar enlargement regions, also entail segmental neuronal loss. Yet, the contributions of gray matter injury and plasticity to functional outcomes are poorly understood. The present study addressed this issue by investigating changes in respiratory function following bilateral C_3/C_4 contusion injuries at the level of the phoenix motoneuron (PhMN) pool which in the adult rat extends from C_3–$C_{5/6}$ and provides innervation to the diaphragm. Despite extensive white and gray matter pathology associated with two magnitudes of injury severity, ventilation was relatively unaffected during both quiet breathing and respiratory challenge (hypercapnia). On the other hand, bilateral diaphragm EMG recordings revealed that the ability to increase diaphragm activity during respiratory challenge was substantially, and chronically, impaired. This deficit has not been seen following predominantly white matter lesions at higher cervical levels. Thus, the impact of gray matter damage relative to PhMNs and/or interneurons becomes evident during conditions associated with increased respiratory drive. Unaltered ventilatory behavior, despite significant deficits in diaphragm function, suggests compensatory neuroplasticity involving recruitment of other spinal respiratory networks which may entail remodeling of connections. Transynaptic tracing, using pseudorabies virus (PRV), revealed changes in PhMN-related interneuronal labeling rostral to the site of injury, thus offering insight into the potential anatomical reorganization and spinal plasticity following cervical contusion.

Keywords

Spinal cord injury; respiration; plasticity; interneuron; pseudorabies virus; contusion

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Introduction

Spinal cord injuries (SCIs) at upper to mid-cervical levels (i.e., C2–C6) often result in long-term impairments in breathing (Sassoon and Baydur, 2003, Winslow and Rozovsky, 2003). Injuries at C4-5 and higher are especially devastating due functional compromise of the diaphragm, which is the main inspiratory muscle. Impaired diaphragm activity is often attributed to interruption of bulbospinal, inspiratory drive projections to PhMNs at C_3 – $C_{5–6}$. However, SCIs at those levels also result in direct uni- or bilateral damage to phoenix motoneurons and their related circuitry (Lane, 2011). Despite its susceptibility to damage, the phoenix motor system, which includes the phoenix nucleus and medullary projections to it, also possesses neuroplastic potential and the capacity for functional adaptations vital for maintaining stable blood gas homeostasis (Goshgarian, 2003, Goshgarian, 2009, Guth, 1976, Minor, et al., 2006). In fact, clinical reports have indicated varying degrees of spontaneous respiratory recovery following high cervical SCI (Bluechardt, et al., 1992, Brown, et al., 2006, Ledsome and Sharp, 1981), and such natural improvements introduce possible opportunities for therapeutically optimizing ventilatory capacity post-SCI.

The most extensively documented example of respiratory dysfunction and neuroplasticity post-SCI to date is the so-called “crossed-phoenix phenomenon” (CPP) (Goshgarian, 2003, Goshgarian, 2009, Lane, et al., 2008a, Lane, et al., 2009, Zimmer, et al., 2007) in which a C_2 lateral hemisection (C_2Hx) results in complete paralysis of the ipsilateral hemidiaphragm. Partial functional recovery can then be induced within hours (Goshgarian, 1979, O’Hara and Goshgarian, 1991) or occur spontaneously weeks after injury (Fuller, et al., 2008, Fuller, et al., 2006, Fuller, et al., 2003, Golder and Mitchell, 2005, Lee, et al., 2010, Nantwi, et al., 1999, Vinit, et al., 2007). Both the C_2Hx and more recent C_2 lateralized (hemi-) contusion (Baussart, et al., 2006) SCI models are predominantly upper motoneuron (i.e., white matter) injuries since the more caudal PhMN pools are completely spared. While providing important proofs-of-concept for neuroplasticity and repair, such injuries are clinically infrequent (NSCISC, 2008) and do not identify with the more common upper and lower motoneuron pathology associated with contusion/compression trauma at the level of the PhMN pool (Lane, 2011, Lane, et al., 2008a).

Three publications to date have explored respiratory function following contusive injuries at the level of the PhMN pool (Choi, et al., 2005b, El-Bohy, et al., 1998a, Golder, et al., 2011) with each showing initially impaired responses to respiratory challenges followed by improvements (Choi, et al., 2005b, Golder, et al., 2011) after lateralized or midline C_4–C_5 contusions. However, the gray versus white matter contributions to either the deficit or improvement remain poorly understood. It also is unclear when respiratory recovery represents restorative versus compensatory neuroplasticity. For the present work, we define “restorative plasticity” as a spontaneous anatomical and/or functional change that reinstates activity within a motor system. In contrast, “compensatory plasticity” encompasses changes in motor systems less directly affected by injury.

The present study examined ventilatory and electrophysiological changes following a mid-cervical ($C_{3/4}$) contusion made at the spinal midline. Changes in the neural substrate mediating phoenix function were also explored using histological and neuroanatomical tracing techniques. The present findings demonstrate that a signature feature of central gray matter damage is an impaired ability to increase diaphragm activity in response to respiratory challenge. Yet, patterns of breathing were unaffected, which is consistent with compensatory plasticity in non-phoenix spinal respiratory circuits.

Methods

Mid-Cervical Spinal Cord Contusions

Adult female Sprague-Dawley rats (215–300 grams, n=52) were obtained from Harlan Scientific and housed at the McKnight Brain Institute Animal Care Facility at the University of Florida. All experimental procedures were conducted with IACUC approval and following NIH guidelines. All animals were anesthetized with xylazine (10mg/kg s.q.) and ketamine (120mg/kg i.p.) and an incision was made from approximately the second to fifth cervical segment (C2-5). Following laminectomy at the C3/4 level, mid-line cervical spinal contusions were made using the Infinite Horizon pneumatic impactor (Precision Systems & Instrumentation, Lexington, KY) (Scheff, et al., 2003). Once the spinal cord was exposed, the impactor probe (2.5mm diameter tip) was raised approximately 5mm above the intact dura, and the cord contused (in air) at a pre-set nominal force of 150 (n=30) or 250 (n=20) kilodynes (KD) (dwell time = 0). The resulting impact forces were 154 (±2) or 228 (±28) KDs, respectively (Table 1) and are subsequently referred to as 150KD and 250KD in the present paper. While all animals survived the injury, only 22 (150KD; 74%) and 7 (250KD; 35%) were retained for functional testing and anatomical analyses. The remaining animals were excluded as they became ventilator dependent (for >1 hour) following injury (see Results).

Upon completion of the injury procedure, overlying muscle and fascia were closed in layers with sterile 4-0 Vicryl suture and the skin incision using sterile wound clips. Animals were then given subcutaneous injections of Lactated Ringers (5ml) to prevent dehydration, yohimbine (1.2mg/kg s.q.) to reverse the action of xylazine, and buprenorphine (~0.05mg/kg s.q.) for analgesia. For plethysmography analysis, injured animals represented their own controls (pre-injury compared against post-injury measurements). For diaphragm EMG (diaEMG) studies, injured animals were compared against recordings from uninjured control animals (n=4). Neuroanatomical tracing results are reported relative to data from uninjured adult rats reported previously (Lane, et al., 2008b).

Blood Oxygen Saturation

Oxygen saturation was monitored throughout the contusion procedure by placing a pulse oximetry probe (MouseOx, Starr Life Sciences Corp.) on the animal's hindpaw. If an animal experienced respiratory arrest or if SpO_2 fell to ≤40%, it was immediately intubated (rat endotracheal tube placed using an otoscope, Hallowell EMC) and mechanically ventilated (Harvard rodent ventilator, Model 683; 70 breaths per minute). Once SpO_2 stabilized above 85% (relatively normal measurements obtained from hindpaw in anesthetized, intubated rats), the animal was weaned from ventilation. If an animal was unable to maintain voluntary breathing, it was euthanized and excluded from the study.

Analysis of Ventilation

As previously described (Fuller, et al., 2008, Fuller, et al., 2006), measures of ventilatory behavior were obtained by whole-body barometric plethysmography (Buxco Inc, Wilmington, NC) from awake, unrestrained animals prior to the spinal contusion procedure and then at weekly intervals for 10 weeks post-injury (n=5 at 150KD and n=4 at 250KD). In addition, a second subset of 150KD animals (n=4) was generated and data obtained pre-injury, and at 1 and 10 weeks post-injury, were compared. Rats were exposed to compressed normoxic gas (21% O_2, balance N_2; flow rate = 2L/min) for an hour of acclimation in the plethysmography chamber, and then exposed to pressurized normoxic, hypercapnic gas mixtures (7% CO_2, 21% O_2, balance N_2; flow rate = 2L/min). The chamber pressure, room temperature, animal body temperature, and barometric pressure were used in the Drorbaugh and Fenn equation to provide a breath-by-breath display of ventilation (Drorbaugh and Fenn,

1955). Parameters which were derived from the airflow traces included inspiratory tidal volume (V_T, ml/100g), respiratory frequency (*f*, breaths/min), minute ventilation ($\dot{V}E$, ml/min/100g), peak inspiratory flow (ml/sec), and peak expiratory flow (ml/sec). Measurements of V_T and $\dot{V}E$ were expressed relative to body weight (Fuller et al., 2008). Following baseline recordings, animals were subjected to a ten minute period of hypercapnic gas (normoxic, hypercapnic (7% CO_2, 21% O_2, balance N_2) inspired air). The purpose of the hypercapnic challenge was to test the ability of the rats to increase ventilation upon demand. This procedure can reveal functional deficits that are not evident during periods of eupnea or "quiet breathing" (Fuller, et al., 2005). Data were averaged from the last ten minutes during baseline and last five minutes during hypercapnic challenge.

Neurophysiology

Terminal bilateral diaEMG recordings were made immediately (n=5), and 1 (n=6) and 12 (n=4) weeks post-contusion just prior to being euthanized and perfusion-fixation for histological analyses (see below). Animals were anesthetized, as described above, and an incision made along linea alba to expose the abdominal surface of the diaphragm. Bipolar hook electrodes (coated tungsten wire with exposed tips) were then inserted into the medial costal region of the left and right hemidiaphragm. Activity was then recorded during spontaneous breathing. Baseline recordings were made by administering compressed air (21% O_2, balance N_2) via a nose cone at a flow rate of 2L/min. Once a stable baseline was attained for a minimum of 10 minutes, the administered air was switched to normoxic, hypercapnic gas (see above) to mirror the challenge used in plethysmography. The diaEMG signals were amplified (1,000×) and band pass filtered (0.3 – 10 KHz) using differential A/C amplifier (Model 1700, A-M Systems) and then full-wave rectified, processed with a moving time averager (time constant = 100ms, Model MA-821RSP, CWE Inc.) and digitized (Power 1401, Cambridge Electronic Design).

Recordings were analysed on a PC computer. The final 1 minute of recording under either baseline or hypercapnia was used for analysis with Spike 2 software (CED) to determine the average change in diaphragm activity in response to hypercapnic challenge (results from hypercapnia as a % of baseline). As all contusions were midline, all recordings from the left and right sides were averaged for each injured and control animal. Average results from uninjured control animals (n=4) were compared against 150KD contused animals 1 week (n=5) and 12 weeks (n=4) post-injury.

Retrograde Anatomical Tracing

Pseudorabies virus (PRV152, Bartha strain) was used to retrogradely label PhMNs and pre-phoenix interneurons. The methods for producing the PRV variant used for this study (PRV152, 6×10^8 pfu/ml) and protocols for diaphragm delivery of the virus and subsequent trans-synaptic tracing were as previously detailed (see Lane, et al., 2008b for technical details of virus production). For the purposes of this investigation, tracing was limited to one hemidiaphragm in order to focus analysis of interneuronal circuitry to only one of the bilaterally damaged PhMN pools. Accordingly, labeled pre-phoenix interneurons were known to innervate this motoneuron pool. In the present study an incision was made along linea alba and the skin and muscles retracted to expose the diaphragm. Tracer was administered to the left half of the diaphragm by topical application (40–50μl). The abdominal muscles were then sutured (4-0 Vicryl), then skin closed with wound clips and animals left to survive for 64–67 hours (n=16).

Anterograde Anatomical Tracing

To label descending fibers from the medullary ventral respiratory column (VRC), biotin dextran amine (BDA, 10,000 kDa) was delivered to physiologically-identified medullary

inspiratory neurons in a subset of animals (n=4) 1 week prior to injury. This approach has been previously described in detail (Lane, et al., 2008b). Briefly, anesthetized animals were placed in a rodent stereotaxic frame (Kopf) and the caudal medulla exposed. A sterile Carbostar-3 electrode (Kation Scientific) was used for electrophysiological recording and tracer delivery. One of the two glass barrels was filled with a 10% solution (w/v in 0.9% sterile saline) of BDA. The electrode was then lowered into the region of the VRC as determined by stereotaxic coordinates (~2mm from the midline and ~0.5 mm rostral to the obex) while recording extracellular activity. Inspiratory cells within the VRC were electrophysiologically identified by observation of rhythmic cellular activity in phase with inspiratory movements of the rib cage which corresponds to diaphragm activity (Lane, et al., 2008b). Once identified, the recording was terminated and the tracer was delivered via iontophoresis over a 30 minute period (5 μA positive current, 7 seconds on / 7 seconds off). The electrode/pipette was then slowly withdrawn, the muscle was sutured in layers and the skin closed with wound clips. Animals were allowed to recover for one week before being euthanized by perfusion-fixation for tissue analysis.

Tissue Fixation

At the end of each experiment, animals were deeply anesthetized with Beuthanasia® (9:1, sodium pentobarbitone to phenytoin solution). For neuroanatomical tracing studies, the animals were intracardially perfused with paraformaldehyde (4% w/v in 0.1M phosphate buffered saline (PBS), pH 7.4). The spinal cords were then removed and stored in paraformaldehyde (2% w/v in PBS). Longitudinal (horizontal) tissue sections were obtained by vibratome sectioning (40μm thick). For more defined histological characterization of these lesions, randomly selected animals were collected immediately (n=1), 1 (n=3), 4 (n=1) and 12 weeks (n=1) post-injury, perfused with paraformaldehyde and glutaraldehyde (4% and 3.5%, respectively, in 0.1 M PBS, pH 7.4) and stored at 4°C for 2 nights. The tissue was then processed and embedded in epoxy resin (Electron Microscopy Sciences, Fort Washington, PA). Sections were cut at 2μm thickness and stained with toluidine blue.

Histological Procedures for Tracing Studies

Longitudinal vibratome sections through the cervical spinal cord were histological stained for the either presence of BDA (anterograde tracing) or immunolabeled with antibodies against PRV (retrograde tracing). Prior to histochemical or immunohistochemical methods, sections were washed in PBS (0.1M (pH=7.4), 3 × 5 minutes), and blocked against endogenous peroxidase activity (30% methanol, 0.6% hydrogen peroxide in 0.1M PBS, incubated for 1 hour).

In tissue from anterograde tracing studies (n=4), the presence of BDA was detected histochemically by incubating sections in a Vectastain ABC solution (Elite Vectastain Kit, Vector Labs) for 2 hours. Sections were then washed and the reaction product was developed with diaminobenzidine (DAB, Sigma). Sections were slide mounted, counterstained with cresyl violet and cover-slipped.

Rabbit anti-PRV (Rb134; provided by Dr. Lynn Enquist, Princeton University, as a service of the National Center for Experimental Neuroanatomy with Neurotropic Viruses: NCRR P40 RRO118604) was used to examine the distribution of PRV labeled PhMNs and pre-phoenix interneurons immediately (n=4), 1 (n=4), 4 (n=4) or 12 weeks (n=4) post-contusion. Detailed characterization of Rb134 and its specificity has been previously described (Lane, et al., 2008b). These sections were also used for quantitative analysis (see below). The optimal dilution of all antibodies for this tissue has been previously determined in our laboratory.

For PRV immunohistochemistry, after blocking against endogenous peroxidase activity sections were re-washed in PBS and blocked against non-specific protein labeling (10% serum in 0.1M PBS with 0.03% Triton-X, incubated for 1 hour). Sections were then incubated at 4°C overnight in Rb134 (1:10,000 - raised against whole, purified PRV particles that were acetone inactivated). The following day, tissue was washed in PBS (0.1M, 3 × 5 minutes), incubated for 2 hours at room temperature in a biotinylated secondary antibody (donkey anti-rabbit, Jackson Immunocytochemicals, 1:200), rewashed in PBS (3 × 5 minutes) and incubated for another 2 hours in Vectastain ABC solution. Sections were then given a third series of washes in PBS and antigen was visualized with DAB. Immunoprecipitation and SDS-page has previously demonstrated that these PRV antibodies are specific for the major capsid, tegument and viral membrane proteins of PRV (Card, et al., 1990, Card, et al., 1991). Immunolabeled sections were then slide mounted, counterstained with cresyl violet and cover-slipped.

Immunohistochemical controls were as previously described (Lane, et al., 2008b). Negative controls included tissue from animals that had not been used for tracing (no antigen present), or that had been used for tracing but where either the primary or secondary antibodies were omitted from the staining protocol. No labeling was observed in negative control tissue sections.

Sections were examined using a Zeiss Axiophot or Axioplan microscope and photographs taken with a digital camera (Axiocam HRc) linked to a computer. For quantification, PRV-positive PhMNs and second-order cells with visible nuclei by brightfield microscopy were counted at 10× magnification in consecutive longitudinal cervical spinal cord sections from each animal. This approach includes only cells with a visible nucleus thereby limiting the possibility of the same cell being counted twice in consecutive sections. To further address over-counting the raw number of cells, an Abercrombie correction was used (T/[T+h], where T = section thickness and h = diameter of the nuclei in the z-axis) (Guillery, 2002). The average diameter of nuclei for each cell population was measured using KS-400 analysis software (KS Imaging Systems, ver. 3.0, Carl Zeiss Vision GmbH). The corrected number of phoenix motoneurons and interneurons are reported as averages for each post-injury time point ± standard deviation.

The number of positively labeled interneurons (secondary labeling) was then also expressed as a function of the number of labeled PhMNs to control for any variation in primary labeling between animals (Lane, et al., 2008b). The resulting ratio is an efficient means of quantifying the relative innervation of phoenix motoneurons by pre-phoenix interneurons and similar approaches have been used in previous studies (Lane, et al., 2008b, Strack and Loewy, 1990).

Statistical Analysis

Results were quantified using Microsoft® Office Excel® 2007 and Spike 2® (version7, Cambridge Electronic Design, UK) software. Average numbers are shown ± standard deviation (SD). Statistical significance was determined using SigmaStat software (Version 3.5, Jandel Scientific). Comparison between treatment groups was performed using ANOVA and Tukey tests (SigmaStat v3.5). All analyses of electrophysiological data were blinded.

Results

Post-Contusion Status and Experimental Inclusion Criteria

Previous reports (e.g. Anderson, et al., 2009a, El-Bohy, et al., 1998b) have differed regarding post-injury survivals and the relative degrees of functional compromise following midline, bilateral contusive SCIs at mid-cervical levels in rats. Therefore, the first objective

of this study was to assess the overall feasibility of this injury model. Intraoperative pulse oximetry was used to noninvasively obtain an index of arterial hemoglobin saturation prior to and after contusion. The mean oxygen saturation (SpO_2) measured from hindpaw in anesthetized prone rats was 88±5% prior to surgery. Following contusion, inspiration was accompanied by vigorous contraction of neck and jaw muscles, and SpO_2 values decreased within 30–60 seconds. Animals that suffered respiratory arrest typically lost voluntary control of breathing ~30 seconds post-injury. Even in those animals that retained spontaneous breathing, SpO_2 rapidly decreased.

Animals were intubated and given assisted ventilation if they suffered respiratory arrest or had post-injury SpO_2 levels <40%. Intubating animals prior to injury was avoided as the introduction of the tube tended to reduce SpO_2 by ~10%. Animals that recovered voluntary control of breathing and maintained a stable SpO_2 >40% were weaned from ventilation within an hour after injury. The present results include only those animals that did not require assisted ventilation beyond 1 hour post-injury.

Of those animals that received a 150KD contusion (n=30), 74% were included in the study. Of those, 33% did not require assisted ventilation, whereas 44% did initially, but then recovered voluntary control of breathing and could be weaned within one hour post-injury. With more severe 250KD contusion (n=20), 35% reached study inclusion criteria. Of these animals, 20% did not require assisted ventilation, and 15% were initially ventilated. Ventilated animals which could not be weaned were immediately euthanized (150KD: 25%, 250KD: 65%).

General Post-injury Health and Neurological Status

Animals resumed feeding and drinking on the same day of surgery. In addition, there were no overt signs of pain or distress (e.g. vocalization) at any time post-injury. Limited weight loss (<20% of pre-surgery body weight) was seen within the first week, but with one exception, animals regained weight by the second week post-surgery. Following 250KD injury, a single animal had delayed weight gain, but then recovered weight by 3 weeks post-injury. The rate of weight gain was less in animals that received the more severe injury.

Motor function was qualitatively evaluated on a daily basis for the first week post-injury and weekly thereafter. In general, the neurological deficits observed and the subsequent pattern of progressive recovery were similar to those described in previous studies of cervical contusion injury (Anderson, et al., 2009a, Choi, et al., 2005a, Gensel, et al., 2006, Pearse, et al., 2005, Schrimsher and Reier, 1992). Animals with either a 150KD or 250KD contusion recovered locomotion 1–2 days post-injury and showed no overt hindlimb deficits (Anderson, et al., 2009b). However, all animals exhibited bilateral forepaw/forelimb impairment which included non-plantar forepaw placement during locomotion and exploring, and abnormal digital and wrist flexion (clenched forepaw) at rest. Forelimb deficits were also underscored by impaired grooming ability within the first 2–3 days post-injury. Within 1–2 weeks post-injury, however, all animals showed some recovery of forelimb function as evidenced by improved forepaw placement during locomotion and recovery of grooming ability.

Midline $C_{3/4}$ Contusions have limited impact on Ventilatory Behavior

In all animals studied up to 10 weeks following either 150KD or 250KD contusion (n=4 in each), ventilation data were obtained pre-injury and at weekly post-injury intervals. There were no statistically significant differences in any pre-injury respiratory parameters between the 150KD and 250 KD animals as determined by whole-body plethysmography. The 150KD contusion had no impact on any of the baseline respiratory parameters (i.e. *f,* V_T,

$\dot{V}E$, PIF and PEF). In addition, these animals showed a robust ventilatory response during hypercapnic challenge (Fig. 1A–C). No significant differences were seen in the relative increase (% baseline) to any of the respiratory parameters between pre- and post-contusion conditions. Ventilatory data obtained prior to and at 1 and 10 weeks following a 150KD injury were obtained from a replicate series of animals (n=4) and showed no differences (P>0.1) thereby supporting consistency of these results.

The more severe contusion (250KD) also had surprisingly little long-term impact on ventilation. The *f* during baseline breathing was similar before and after contusion. We noted a tendency for a blunted increase in *f* during hypercapnia, but this did not reach statistical significance (P=0.2) (Fig. 1D). However, when expressed relative to pre-injury values (% pre-injury) the hypercapnic *f* response was reduced for the first three weeks post-injury (P<0.05, data not shown). Following the initial decline, hypercapnic *f* values tended to rise over subsequent weeks until reaching a plateau by 10 weeks that approximated pre-injury values. Despite the widespread neuropathology associated with 250KD injury (Fig. 3), the baseline inspiratory V_T was significantly increased only at the first week post-injury (P<0.01) (Fig. 1E). While not significantly greater than pre-injury values, the V_T remained elevated until 4 weeks post-injury when it returned to pre-injury values. Hypercapnia V_T was not overtly affected by contusion. Overall there were no robust effects on $\dot{V}E$ following 250 KD injuries during baseline breathing or hypercapnic challenge. No differences were seen in the mean percent-response to challenge, pre-injury and 1, 4 and 10 weeks post-injury.

Long-term Diaphragm EMG Activity Following Associated with Midline C3/4 Contusion

Diaphragm function was assessed by bilateral diaEMG recordings during spontaneous eupneic breathing or hypercapnic challenge (7% CO_2) in anesthetized animals (Fig. 2). A robust increase in diaphragm activity during hypercapnic challenge is a characteristic respiratory feature in uninjured animals (n=4, Fig. 2A). Immediately following 150KD injury (n=5), there was a loss of rhythmic diaphragm activity in anesthetized, spontaneously breathing animals, under baseline (room air) conditions. One week following 150KD contusion (n=6), diaphragm activity was observed bilaterally in all animals, but when compared to uninjured rats, the ability to increase EMG bursting during hypercapnia was significantly reduced (P<0.001; Fig. 2C). The average response to challenge was just 114 ±14% of baseline activity. Similarly, while baseline activity was maintained bilaterally at 12 weeks post-injury (n=4), the response to hypercapnia remained minimal with an average increase of 114 ±13% of baseline. Thus, the 150KD cervical contusion resulted in a persistent impairment in the animal's ability to recruit diaphragm motor units and/or increase bursting in active motor units in response to a substantial chemical challenge.

Histopathology

The location of injury in all animals was macroscopically confirmed to be at the level of caudal C3/rostral C4. Histological examination of the lesion epicenters of 150 and 250KD injured animals revealed extensive central gray matter necrosis which entailed bilateral damage to ventral gray matter in the region of the phoenix nucleus (Figs. 3, 5, 6). The overall pathology of these injuries (Fig. 3) was otherwise similar to what has been extensively described relative to experimental SCIs in general (e.g., Cao, et al., 2005, Reier, 2004; see also description in Figure 3). One notable difference was the absence of obvious primary demyelination, which has been reported by others in relation to mid-thoracic contusions in cat and rat (Blight, 1985, Cao, et al., 2005, Totoiu and Keirstead, 2005), but not mouse (Lasiene, et al., 2008). Histological analysis of plastic sections of tissue from animals that received a 150KD injury (n=3) revealed various stages of axonal degeneration and secondary demyelination (i.e., Wallerian degeneration) throughout the dorsal, lateral,

ventrolateral and ventromedial white matter (Fig. 4A) but profiles suggestive of spared, denuded axons were not observed.

Cystic cavitation occurred at the lesion epicenter, encompassing dorsal, intermediate and ventral gray matter, in which extensive monocyte infiltration was visible. The substantia gelatinosa and some immediately underlying dorsal gray matter remained intact and showed relatively modest pathology. Minimal, if any, tissue sparing also was seen in the far lateral portions of ventral gray matter on one or both sides of the lesion epicenter There was little change in general lesion morphology 12 weeks post-injury, as has been reported previously by others (Noble and Wrathall, 1989).

Bulbospinal Projections from Inspiratory Neurons following 150KD Injury

To determine the effect of 150KD contusion on bulbospinal projections from the VRC, biotin dextran amine (BDA) was iontophoretically delivered to physiologically identified inspiratory neurons in the medulla (Lane, et al., 2008b). Qualitatively, tissue sections at one week following injury (2 weeks post-BDA delivery), showed numerous bouton-like profiles around ventral horn neurons in the region of PhMNs (based on previously published observations (Lane, et al., 2008b)), rostral to the lesion (Fig. 5A). As shown in uninjured animals, boutons were present around neuronal cell soma and in the immediate surrounding area (known to be high in dendritic density (Lane, et al., 2008b). Toward the rostral edge of the cystic cavity, beaded axonal profiles (indicative of degeneration of labeled axons) were visible (Fig. 5B). Labeled axons with signs of compromise also were seen at the level of injury in the lateral and ventromedial white matter. Labeled axons were sparse in all regions of gray matter caudal to the lesion (Fig. 5C).

Phoenix Motoneurons and Pre-phoenix Interneurons following 150KD injury

Immediately following 150KD contusion—Retrograde, transynaptic pre-labeling of the phoenix circuit illustrated the loss of both motoneurons and interneurons following 150KD contusion. Delivery of PRV to the left hemidiaphragm 64 hours prior to injury (n=4), revealed the distribution (Fig. 5) and raw number of PhMNs labeled immediately post-contusion (168±35) was comparable to that previously reported in uninjured adult female rats (Lane, et al., 2008b). The lesion epicenter was visible in this tissue as being edematous with parenchymal irregularity and bleeding (Fig. 5). All pre-labeled cells within the lesion epicenter showed signs of pathology (e.g. cytoplasmic disruption, Fig. 5B–C). Analysis of the number of PhMNs revealed that an average of 53 ±20% of the PhMN pool was located within the region of edema (Fig 5D). Neurons immediately adjacent to regions of tissue edema, either rostral or caudal to the injury site, did not show any overt signs of pathology. Labeled motoneurons were observed both rostral and caudal to injury. While the proportion of motoneurons in these regions varied in each animal, the averages were comparable (29 ±2% and 19 ±22% respectively).

The total number pre-phoenix interneurons that were pre-labeled ipsi- and contralateral to the labeled PhMN pool was 98 (±25) and 21 (±12), respectively, The ratio of labeled interneurons, ipsi- and contralateral to labeled PhMNs, was approximately 43 (±21)% and 6(±8)% respectively. This number is comparable to that previously reported in uninjured animals (Lane, et al., 2008b). Approximately one-third of PRV labeled pre-phoenix interneurons were observed within the lesion epicenter immediately post-contusion. As was the case for PhMNs, the proportion of spared pre-labeled interneurons was evenly distributed between the cervical cord rostral and caudal to the injury.

1–12 weeks following 150KD contusion—While there is a growing use of PRV to transynaptically trace neuronal connectivity following SCI (Bareyre, et al., 2004, Duale, et

al., 2009, Duale, et al., 2010, Im, et al., 2008, Kim, et al., 2002, Lane, et al., 2009, Lane, et al., 2008b, Yu, et al., 2003), we (Lane, et al., 2009) and others (Duale, et al., 2009, Duale, et al., 2010) have reported a reduction in the extent of first-order PRV neuronal infection as a function of time after SCI. In other on-going studies (unpublished), we have determined that the rate of infection is delayed such that maximum first-order labeling of PhMNs occurs 24 hours later than normal.

For this study, however, we continued to apply the 64hr second-order survival schedule since it provides a conservative index of circuitry changes, and found there was already a reduction in the number of labeled PhMNs at one week post-injury (n=4) (Fig. 6). An average of 139 (±72) infected PhMNs were observed 1 week post-injury, compared with 168±35 pre-labeled cells observed immediately post-injury. The majority of labeling 1 week post-injury (75 ±18%) was present rostral to the lesion epicenter, compared with 29 ±2% in animals examined immediately post-injury ($P=0.01$), the number of cells observed caudal to the lesion was significantly reduced ($P<0.05$). The extent of PhMN labeling was further reduced by 4 (n=4) and 12 (n=4) weeks post-contusion ($P<0.05$), with no detectable labeling observed in 3 of the 4 animals in each group.

The raw number of labeled interneurons was not significantly different between animals examined immediately (81 ±68) and 1 week post-injury (219 ±88). Despite variability in labeling seen between animals, 1 week post-injury there was a significant increase in the ratio of interneurons to motoneurons observed rostral to the injury ($P<0.05$). One week post-injury approximately 60 ±12% and 55 ±19% of interneurons ipsi- and contralateral to the phoenix pool were detected rostral to the injury site in the ventral horn and medial gray matter. The ratio of interneurons ipsi- and contralateral to labeled PhMNs 1 week post injury was 117 (±33)% and 37 (±8)%, respectively (Fig. 6D). The distribution of labeled interneurons, and their number relative to labeled motoneurons, thus appeared to be altered relative to the labeling pattern seen immediately post-injury.

Discussion

While there is considerable emphasis on neuroprotection (Kwon, et al., 2010, Tator and Fehlings, 1999) and potential need for motoneuron replacement after SCI (Clowry, et al., 1991, Clowry and Vrbova, 1992, Erceg, et al., 2011, Rossi, et al., 2011), the relative contributions of gray versus white matter damage to functional outcomes in clinically-relevant contusion injury models is virtually unknown. Some evidence indicates, however, that focal excitotoxic deletion of spinal gray matter alone can have profound impact on locomotion (Hadi, et al., 2000, Magnuson, et al., 1999) and manifestation of pain (Yezierski, et al., 1998). The present results demonstrate that chronic attenuation of diaEMG activity in response to respiratory challenge is a signature outcome of midline contusion injuries at the level of the PhMN pool. By comparison with predominant white matter pathology and respiratory outcomes in other mid-to-high cervical SCI models (Table 2), the present findings suggest a functionally distinct effect of gray matter loss on post-SCI diaphragm function, similar to those reported in humans. Despite compromised diaphragm activity, ventilatory responses to hypercapnia were remarkably unaffected even with increased injury severity. Accordingly, our data suggest a robust engagement of compensatory function, presumably in other spinal respiratory circuits, to maintain appropriate patterns of breathing behavior.

Respiratory Dysfunction associated with Upper & Lower Motoneuron Compromise

White matter disruption—Of the models used to study respiration after SCI, C_2Hx results in the most extensive direct white matter damage and unilateral denervation of the phoenix nucleus. That pathology results in an initial silencing of phoenix nerve activity

(Fuller, et al., 2008, Fuller, et al., 2006, Golder, et al., 2001a, O'Hara and Goshgarian, 1991) and paralysis of the ipsilateral hemidiaphragm (Guth, 1976, Nantwi, et al., 1999, Vinit, et al., 2006). Less severe deficits also occur in ipsilateral phoenix output and diaphragm activity following incomplete lateralized C_2Hx or contusions (Baussart, et al., 2006, El-Bohy, et al., 1998b, Fuller, et al., 2009, Vinit, et al., 2006). The degree of hemidiaphragm impairment appears to be a function of the extent of the bulbospinal-PhMN disconnection.

White matter compromise also can have profound effects on breathing behavior (see Table 2) as C_2Hx leads to an increased respiratory frequency and decreased V_T (i.e., rapid, shallow breathing (Fuller, et al., 2008, Fuller, et al., 2006, Fuller, et al., 2009, Golder, et al., 2001b, Goshgarian, et al., 1986)). Our plethysmography data thus differ from those reported by Choi et al (2005) who observed changes in the pattern of breathing behavior for at least 3 weeks following graded C5 lateralized contusion injuries. The degree of ipsilateral white matter damage in that study was comparatively greater than produced by midline injuries in the present work. In general, ventilatory outcomes following severe lateralized contusions appear to more closely parallel those seen with extensive white matter damage in lateral hemisection models (Table 2). Therefore, white matter sparing, even following more severe (250KD) midline contusions, most likely explains why eupneic breathing was only temporarily affected in the present study.

Gray matter disruption—Bilateral diaEMG recordings in the present work revealed attenuated diaphragm activity in response to respiratory challenge at one week post-injury (150KD). This deficit was similar at 12 weeks post-injury. Consistent with these results, El-Bohy et al. (1998b) reported minimal differences in phoenix output during baseline or challenge (asphyxia), 2–11 weeks following midline C4/5 contusion (NYU spinal contusion device). A recent study (Golder et al., 2011) also showed diminished capacities to increase phoenix burst amplitudes following hypoxic or hypercapnic challenges in rats with predominantly lateralized C4-5 contusions (NYU device). In contrast, such a deficit is absent in models focused primarily on white matter compromise (complete or partial C_2Hx, Table 2).

A consistent histopathological feature of C_3–C_5 contusions is midline destruction of intermediate gray matter with expansion to one or both PhMN pools depending upon the nature or severity of the injury (lateralized versus midline) and symmetry of the lesion (Table 2). Although we did not quantify the extent of cell death, the retrograde labeling data suggest vulnerability of >50% of the PhMN pool. Choi et al. (2005) attributed the ventilatory dysfunction they observed to an injury-dependent loss of ventral horn cells though PhMN attrition was not specifically determined. A similar speculation was proposed by Golder et al. (2011) for changes in tidal volume and phoenix nerve burst amplitudes which they reported. However, in the present study PhMN loss did not appear to overtly affect ventilation, whereas the impaired diaphragm response to challenge is likely to be more directly related. Pathophysiological interruption of the rostral-caudal continuity of the PhMN column and its unique longitudinal dendro-dendritic architecture and connectivity (Goshgarian and Rafols, 1981, Lane, 2011, Torikai, et al., 1996) may also result in altered recruitment of previously silent neurons (Milano, et al., 1992, St John and Bartlett, 1979) and rate coding of active cells (Lee and Fuller, 2011, Lee, et al., 2009). For example, if a considerable portion of the phoenix motor pool is lost following contusion, as suggested by PRV labelling (see Fig. 5), surviving but previously silent cells may be recruited in an effort to maintain effective diaphragm contraction under baseline breathing conditions. However, in this scenario, further increases in diaphragm EMG activity (e.g. during respiratory challenge) would be limited (Fig. 2B) (Golder et al., 2011). While changes in PhMN recruitment have been studied following C_2Hx injuries (El-Bohy and Goshgarian, 1999, Mantilla and Sieck, 2009, Sieck and Mantilla, 2009) the impact of chronic cervical

contusion injury on these fundamental mechanisms remains unknown. In addition, PhMN recruitment during respiratory challenge may be affected to some extent by reduced bulbospinal innervation of spared PhMN neurons caudal to the injury as our anterograde VRC tracing results suggest.

Ventilatory insufficiency following contusions at C_3–C_5, however, may involve more than PhMN loss per se as such injuries also affect intermediate gray matter and constituent propriospinal neuron populations (Conta-Steencken, et al., 2009, Conta-Steencken and Stelzner, 2010, Conta and Stelzner, 2004). Transneuronal tracing results indicate potential vulnerability of >30% of pre-phoenix interneurons. Because interneurons throughout the cervical spinal cord have been shown to innervate both phoenix and intercostal motoneurons (Bellingham and Lipski, 1990, Billig, et al., 2000, Lane, et al., 2009, Lane, et al., 2008b, Lipski, et al., 1993), loss of these cells or changes in their connectivity may influence post-injury PhMN responses leading to reduced diaphragm activity during increased respiratory drive. Consideration of pre-phoenix interneuron loss is further motivated by our initial extracellular single-unit recording studies which have shown that some neurons in mid-cervical intermediate gray matter are responsive to respiratory challenge (Lane, et al., 2009).

Establishing changes in interneuronal connectivity post-contusion is a challenging, yet exceptionally important aspect of SCI research given the role of spinal interneurons in spontaneous anatomical plasticity and functional recovery (Bareyre, et al., 2004, Courtine, et al., 2008, Flynn, et al., 2011, Stelzner, 2008). Interneurons also play a key role in shaping motoneuron output, including modulation of phoenix activity following cervical SCI (Alilain, et al., 2008, Alilain and Silver, 2009, Hayashi, et al., 2003, Lane, et al., 2009, Sandhu, et al., 2009). It is thus intriguing that our trans-synaptic tracing experiments revealed an altered pattern of interneuronal PRV labeling rostral to the lesion epicenter. This may reflect either new neurons being recruited into the phoenix circuitry or increased sprouting and earlier labeling of established pre-phoenix interneurons. Further neuroanatomical and electrophysiological studies are needed to test the functional significance of such neural circuit remodeling.

Compensatory Respiratory Plasticity following Mid-Cervical Spinal Contusions

Respiratory neuroplasticity has been extensively demonstrated in the C_2Hx model (Goshgarian, 2009, Lane, et al., 2008a, Lane, et al., 2009, see also Alilain et al. in this issue) and attributed not only to spontaneous restoration of activity to the diaphragm ipsilateral to injury (“restorative plasticity”), but concomitant “compensatory plasticity” as well (Dougherty, et al., 2011, Golder, et al., 2003, Lee, et al., 2010). Although previous studies of respiratory function post-SCI have revealed significant deficits in ventilation, the present study showed that with one transitory exception, midline mid-cervical contusions had limited impact on breathing behavior. Though tidal volume during eupneic breathing was increased one week following 250KD contusion, even that effect was resolved by 2 weeks post-injury. As discussed above, this may reflect recruitment of previously silent PhMNs and thus functional plasticity within the phoenix spinal circuit. Choi et al (2005a) reported ventilatory recovery within weeks following lateralized contusions, which the authors attributed to phoenix plasticity involving sprouting and reinnervation of the hemidiaphragm by spared ipsilateral or contralateral PhMNs (Choi, et al., 2005a). The current findings raise an alternative interpretation since relatively normal ventilatory responses to hypercapnia were seen in the absence of any significant increase in diaphragm activity. This remarkable outcome in post-injury breathing behavior cannot be attributed to improved phoenix function or diaphragm recovery (Choi et al., 2005; Golder et al., 2011). Instead, the current data suggest that the post-injury breathing patterns seen here and the ventilatory recoveries reported in the Choi and Golder studies most likely reflect compensatory plasticity in non-phoenix respiratory systems (e.g. intercostal and/or accessory respiratory muscle groups).

Translational Implications

Pre-clinical animal modeling of a long-term, ventilator-dependent individual is not currently feasible for practical and biological reasons (Levine, et al., 2008, Powers, et al., 2009, Powers, et al., 2002, Shanely, et al., 2004, Sieck and Mantilla, 2008). Nevertheless, models of less severe deficits may be comparable to patients who are weaned from mechanical ventilation, but who still exhibit significantly compromised respiratory function (e.g., induced by challenging conditions as simple as the common cold). In many respects, outcomes of mid-cervical contusion models described herein identify with that significant portion of the cervical SCI population. The inability of the respiratory neuromuscular system to increase output in response to a physiological challenge is a featured, though not exclusive, result of experimental mid-cervical contusion injuries. From a preclinical perspective, this is a reproducible (and thus robust) experimental outcome of cervical contusion injuries which has now been documented using ventilatory and electrophysiological outcome measures (El-Bohy et al., 1998; Choi et al,, 2005, Golder et al., 2011, and the present study). Importantly, a similar chronic deficit has been described in quadriplegic patients (Kelling, et al., 1985, Lin, et al., 1998, Manning, et al., 1992) when presented with hypercapnic or hypoxic respiratory challenge.

The underlying neural basis remains to be determined, although current and previously reported data (Choi, et al., 2005a) indicate a significant gray matter involvement especially when contrasted with injuries primarily affecting white matter. Indeed, respiratory responses to challenge are compromised to a much lesser extent by chronic C2Hx injuries (Fuller, et al., 2008). Further studies and refinements of experimental cervical contusion injury models can be instrumental in defining future therapeutic approaches aimed at gray matter repair. For example, various cellular replacement (Clowry, et al., 1991, Reier, 2004, White, et al., 2010) and selective lesion (Hadi, et al., 2000, Magnuson, et al., 1999, Magnuson, et al., 2001) approaches have potential for determining the relative contributions of PhMN and interneuronal loss or reorganization to post-injury respiratory function and the blunted phoenix responses to challenge reported here.

“Neuroplasticity” is a term rarely included in clinical discussions related to respiratory dysfunction following SCI. Yet after ventilator weaning, patients achieve eupneic breathing by alternative behaviors involving accessory respiratory muscles (Ledsome and Sharp, 1981, Winslow and Rozovsky, 2003) and their respective circuitries. Presently, very little is known about the neurobiology underlying such compensatory neuroplasticity especially with regard to mid-cervical contusions. This is of particular interest since such injuries will most likely damage a key neuroanatomical substrate believed to mediate the most extensively documented form of post-SCI respiratory plasticity (Goshgarian, 2003, Goshgarian, 2009, Lane, et al., 2008a). Insights into the neuroplastic features of bulbospinal respiratory circuits from anatomical, neurophysiological and molecular perspectives may ultimately generate greater focus on rehabilitation (Martin, et al., 2011)and other novel approaches (DiMarco, 2009, Khong, et al., 2010) for improving breathing performance after cervical SCI.

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Abbreviations

BDA	biotin dextran amine
C#	spinal cervical segment #
C_2Hx	C2 lateral hemisection
CPP	crossed-phoenix phenomenon
diaEMG	diaphragm electromyography
f	breathing frequency
KD	kilodynes
PEF	peak expiratory flow
PhMN	phoenix motoneuron
PIF	peak inspiratory flow
PRV	pseudorabies virus
SpO2	blood oxygen saturation
$\dot{V}$E	minute ventilation
VRC	ventral respiratory column
V_T	tidal volume

References

1. Alilain WJ, Li X, Horn KP, Dhingra R, Dick TE, Herlitze S, Silver J. Light-induced rescue of breathing after spinal cord injury. J Neurosci. 2008; 28:11862–11870. [PubMed: 19005051]
2. Alilain WJ, Silver J. Shedding light on restoring respiratory function after spinal cord injury. Front Mol Neurosci. 2009; 2:18. [PubMed: 19893756]
3. Anderson KD, Sharp KG, Steward O. Bilateral cervical contusion spinal cord injury in rats. Exp Neurol. 2009a
4. Anderson KD, Sharp KG, Steward O. Bilateral cervical contusion spinal cord injury in rats. Exp Neurol. 2009b; 220:9–22. [PubMed: 19559699]
5. Bareyre FM, Kerschensteiner M, Raineteau O, Mettenleiter TC, Weinmann O, Schwab ME. The injured spinal cord spontaneously forms a new intraspinal circuit in adult rats. Nat Neurosci. 2004; 7:269–277. [PubMed: 14966523]
6. Baussart B, Stamegna JC, Polentes J, Tadie M, Gauthier P. A new model of upper cervical spinal contusion inducing a persistent unilateral diaphragmatic deficit in the adult rat. Neurobiol Dis. 2006; 22:562–574. [PubMed: 16488616]
7. Bellingham MC, Lipski J. Respiratory interneurons in the C5 segment of the spinal cord of the cat. Brain Res. 1990; 533:141–146. [PubMed: 2085725]
8. Billig I, Foris JM, Enquist LW, Card JP, Yates BJ. Definition of neuronal circuitry controlling the activity of phoenix and abdominal motoneurons in the ferret using recombinant strains of pseudorabies virus. J Neurosci. 2000; 20:7446–7454. [PubMed: 11007904]
9. Blight AR. Delayed demyelination and macrophage invasion: a candidate for secondary cell damage in spinal cord injury. Cent Nerv Syst Trauma. 1985; 2:299–315. [PubMed: 3836014]
10. Bluechardt MH, Wiens M, Thomas SG, Plyley MJ. Repeated measurements of pulmonary function following spinal cord injury. Paraplegia. 1992; 30:768–774. [PubMed: 1484727]
11. Brown R, DiMarco AF, Hoit JD, Garshick E. Respiratory dysfunction and management in spinal cord injury. Respir Care. 2006; 51:853–868. discussion 869–870. [PubMed: 16867197]

12. Cao Q, Zhang YP, Iannotti C, DeVries WH, Xu XM, Shields CB, Whittemore SR. Functional and electrophysiological changes after graded traumatic spinal cord injury in adult rat. Exp Neurol. 2005; 191(Suppl 1):S3–S16. [PubMed: 15629760]
13. Card JP, Rinaman L, Schwaber JS, Miselis RR, Whealy ME, Robbins AK, Enquist LW. Neurotropic properties of pseudorabies virus: uptake and transneuronal passage in the rat central nervous system. J Neurosci. 1990; 10:1974–1994. [PubMed: 2162388]
14. Card JP, Whealy ME, Robbins AK, Moore RY, Enquist LW. Two alpha-herpesvirus strains are transported differentially in the rodent visual system. Neuron. 1991; 6:957–969. [PubMed: 1711350]
15. Choi H, Liao WL, Newton KM, Onario RC, King AM, Desilets FC, Woodard EJ, Eichler ME, Frontera WR, Sabharwal S, Teng YD. Respiratory abnormalities resulting from midcervical spinal cord injury and their reversal by serotonin 1A agonists in conscious rats. J Neurosci. 2005a; 25:4550–4559. [PubMed: 15872102]
16. Choi H, Liao WL, Newton KM, Onario RC, King AM, Desilets FC, Woodard EJ, Eichler ME, Frontera WR, Sabharwal S, Teng YD. Respiratory abnormalities resulting from midcervical spinal cord injury and their reversal by serotonin 1A agonists in conscious rats. J Neurosci. 2005b; 25:4550–4559. [PubMed: 15872102]
17. Clowry G, Sieradzan K, Vrbova G. Transplants of embryonic motoneurones to adult spinal cord: survival and innervation abilities. Trends Neurosci. 1991; 14:355–357. [PubMed: 1721741]
18. Clowry GJ, Vrbova G. Observations on the development of transplanted embryonic ventral horn neurones grafted into adult rat spinal cord and connected to skeletal muscle implants via a peripheral nerve. Exp Brain Res. 1992; 91:249–258. [PubMed: 1459227]
19. Conta-Steencken AC, Siebert JR, Stelzner DJ. Lack of axonal sprouting of spared propriospinal fibers caudal to spinal contusion injury is attributed to chronic axonopathy. J Neurotrauma. 2009; 26:2279–2297. [PubMed: 19645528]
20. Conta-Steencken AC, Stelzner DJ. Loss of propriospinal neurons after spinal contusion injury as assessed by retrograde labeling. Neuroscience. 2010; 170:971–980. [PubMed: 20659532]
21. Conta AC, Stelzner DJ. Differential vulnerability of propriospinal tract neurons to spinal cord contusion injury. J Comp Neurol. 2004; 479:347–359. [PubMed: 15514981]
22. Courtine G, Song B, Roy RR, Zhong H, Herrmann JE, Ao Y, Qi J, Edgerton VR, Sofroniew MV. Recovery of supraspinal control of stepping via indirect propriospinal relay connections after spinal cord injury. Nat Med. 2008; 14:69–74. [PubMed: 18157143]
23. DiMarco AF. Phoenix nerve stimulation in patients with spinal cord injury. Respir Physiol Neurobiol. 2009; 169:200–209. [PubMed: 19786125]
24. Dougherty BJ, Lee KZ, Lane MA, Reier PJ, Fuller DD. The contribution of the spontaneous crossed-phoenix phenomenon to inspiratory tidal volume. J Appl Physiol. 2011 In press.
25. Drorbaugh JE, Fenn WO. A barometric method for measuring ventilation in newborn infants. Pediatrics. 1955; 16:81–87. [PubMed: 14394741]
26. Duale H, Hou S, Derbenev AV, Smith BN, Rabchevsky AG. Spinal cord injury reduces the efficacy of pseudorabies virus labeling of sympathetic preganglionic neurons. J Neuropathol Exp Neurol. 2009; 68:168–178. [PubMed: 19151624]
27. Duale H, Lyttle TS, Smith BN, Rabchevsky AG. Noxious colorectal distention in spinalized rats reduces pseudorabies virus labeling of sympathetic neurons. J Neurotrauma. 2010; 27:1369–1378. [PubMed: 20528165]
28. El-Bohy AA, Goshgarian HG. The use of single phoenix axon recordings to assess diaphragm recovery after cervical spinal cord injury. Exp Neurol. 1999; 156:172–179. [PubMed: 10192788]
29. El-Bohy AA, Schrimsher GW, Reier PJ, Goshgarian HG. Quantitative assessment of respiratory function following contusion injury of the cervical spinal cord. Exp Neurol. 1998a; 150:143–152. [PubMed: 9514833]
30. El-Bohy AA, Schrimsher GW, Reier PJ, Goshgarian HG. Quantitative assessment of respiratory function following contusion injury of the cervical spinal cord. Exp Neurol. 1998b; 150:143–152. [PubMed: 9514833]
31. Erceg S, Ronaghi M, Oria M, Rosello MG, Arago MA, Lopez MG, Radojevic I, Moreno-Manzano V, Rodriguez-Jimenez FJ, Bhattacharya SS, Cordoba J, Stojkovic M. Transplanted

oligodendrocytes and motoneuron progenitors generated from human embryonic stem cells promote locomotor recovery after spinal cord transection. Stem Cells. 2011; 28:1541–1549. [PubMed: 20665739]

32. Flynn JR, Graham BA, Galea MP, Callister RJ. The role of propriospinal interneurons in recovery from spinal cord injury. Neuropharmacology. 2011; 60:809–822. [PubMed: 21251920]
33. Fuller, DD.; Bavis, RW.; Mitchell, GS. Respiratory Neuroplasticity: Respiratory Gases, Development, and Spinal Injury. In: Ward, DS.; Dahan, A.; Teppema, L., editors. Pharmacology and Pathophysiology of the Control of Breathing. Taylor & Francis; Boca Raton: 2005. p. 155-223.
34. Fuller DD, Doperalski NJ, Dougherty BJ, Sandhu MS, Bolser DC, Reier PJ. Modest spontaneous recovery of ventilation following chronic high cervical hemisection in rats. Exp Neurol. 2008; 211:97–106. [PubMed: 18308305]
35. Fuller DD, Golder FJ, Olson EB Jr, Mitchell GS. Recovery of phoenix activity and ventilation after cervical spinal hemisection in rats. J Appl Physiol. 2006; 100:800–806. [PubMed: 16269524]
36. Fuller DD, Johnson SM, Olson EB Jr, Mitchell GS. Synaptic pathways to phoenix motoneurons are enhanced by chronic intermittent hypoxia after cervical spinal cord injury. J Neurosci. 2003; 23:2993–3000. [PubMed: 12684486]
37. Fuller DD, Sandhu MS, Doperalski NJ, Lane MA, White TE, Bishop MD, Reier PJ. Graded unilateral cervical spinal cord injury and respiratory motor recovery. Respir Physiol Neurobiol. 2009; 165:245–253. [PubMed: 19150658]
38. Gensel JC, Tovar CA, Hamers FP, Deibert RJ, Beattie MS, Bresnahan JC. Behavioral and histological characterization of unilateral cervical spinal cord contusion injury in rats. J Neurotrauma. 2006; 23:36–54. [PubMed: 16430371]
39. Golder FJ, Fuller DD, Davenport PW, Johnson RD, Reier PJ, Bolser DC. Respiratory motor recovery after unilateral spinal cord injury: eliminating crossed phoenix activity decreases tidal volume and increases contralateral respiratory motor output. J Neurosci. 2003; 23:2494–2501. [PubMed: 12657710]
40. Golder FJ, Fuller DD, Lovett-Barr MR, Vinit S, Resnick DK, Mitchell GS. Breathing patterns after mid-cervical spinal contusion in rats. Experimental neurology. 2011
41. Golder FJ, Mitchell GS. Spinal synaptic enhancement with acute intermittent hypoxia improves respiratory function after chronic cervical spinal cord injury. J Neurosci. 2005; 25:2925–2932. [PubMed: 15772352]
42. Golder FJ, Reier PJ, Bolser DC. Altered respiratory motor drive after spinal cord injury: supraspinal and bilateral effects of a unilateral lesion. J Neurosci. 2001a; 21:8680–8689. [PubMed: 11606656]
43. Golder FJ, Reier PJ, Davenport PW, Bolser DC. Cervical spinal cord injury alters the pattern of breathing in anesthetized rats. J Appl Physiol. 2001b; 91:2451–2458. [PubMed: 11717204]
44. Goshgarian H. The crossed phoenix phenomenon: a model for plasticity in the respiratory pathways following spinal cord injury. J Appl Physiol. 2003; 94:795–810. [PubMed: 12531916]
45. Goshgarian HG. Developmental plasticity in the respiratory pathway of the adult rat. Exp Neurol. 1979; 66:547–555. [PubMed: 488237]
46. Goshgarian HG. The crossed phoenix phenomenon and recovery of function following spinal cord injury. Respir Physiol Neurobiol. 2009
47. Goshgarian HG, Moran MF, Prcevski P. Effect of cervical spinal cord hemisection and hemidiaphragm paralysis on arterial blood gases, pH, and respiratory rate in the adult rat. Exp Neurol. 1986; 93:440–445. [PubMed: 3089833]
48. Goshgarian HG, Rafols JA. The phoenix nucleus of the albino rat: a correlative HRP and Golgi study. J Comp Neurol. 1981; 201:441–456. [PubMed: 7276259]
49. Guillery RW. On counting and counting errors. J Comp Neurol. 2002; 447:1–7. [PubMed: 11967890]
50. Guth L. Functional plasticity in the respiratory pathway of the mammalian spinal cord. Exp Neurol. 1976; 51:414–420. [PubMed: 1269570]

51. Hadi B, Zhang YP, Burke DA, Shields CB, Magnuson DS. Lasting paraplegia caused by loss of lumbar spinal cord interneurons in rats: no direct correlation with motor neuron loss. J Neurosurg. 2000; 93:266–275. [PubMed: 11012058]
52. Hayashi F, Hinrichsen CF, McCrimmon DR. Short-term plasticity of descending synaptic input to phoenix motoneurons in rats. J Appl Physiol. 2003; 94:1421–1430. [PubMed: 12482770]
53. Im YJ, Hong CH, Jin MH, Lee BH, Han SW. c-fos expression in bladder-specific spinal neurons after spinal cord injury using pseudorabies virus. Yonsei Med J. 2008; 49:479–485. [PubMed: 18581599]
54. Kelling JS, DiMarco AF, Gottfried SB, Altose MD. Respiratory responses to ventilatory loading following low cervical spinal cord injury. J Appl Physiol. 1985; 59:1752–1756. [PubMed: 4077783]
55. Khong P, Lazzaro A, Mobbs R. Phoenix nerve stimulation: The Australian experience. J Clin Neurosci. 2010
56. Kim ES, Kim GM, Lu X, Hsu CY, Xu XM. Neural circuitry of the adult rat central nervous system after spinal cord injury: a study using fast blue and the Bartha strain of pseudorabies virus. J Neurotrauma. 2002; 19:787–800. [PubMed: 12165138]
57. Kwon BK, Okon EB, Hillyer J, Mann C, Baptiste DC, Weaver L, Fehlings M, Tetzlaff W. A Systematic Review of Non-Invasive Pharmacologic Neuroprotective Treatments for Acute Spinal Cord Injury. J Neurotrauma. 2010
58. Lane MA. Spinal respiratory motoneurons and interneurons. Respir Physiol Neurobiol. 2011
59. Lane MA, Fuller DD, White TE, Reier PJ. Respiratory neuroplasticity and cervical spinal cord injury: translational perspectives. Trends Neurosci. 2008a; 31:538–547. [PubMed: 18775573]
60. Lane MA, Lee KZ, Fuller DD, Reier PJ. Spinal circuitry and respiratory recovery following spinal cord injury. Respir Physiol Neurobiol. 2009; 169:123–132. [PubMed: 19698805]
61. Lane MA, White TE, Coutts MA, Jones AL, Sandhu MS, Bloom DC, Bolser DC, Yates BJ, Fuller DD, Reier PJ. Cervical prephrenic interneurons in the normal and lesioned spinal cord of the adult rat. J Comp Neurol. 2008b; 511:692–709. [PubMed: 18924146]
62. Lasiene J, Shupe L, Perlmutter S, Horner P. No evidence for chronic demyelination in spared axons after spinal cord injury in a mouse. J Neurosci. 2008; 28:3887–3896. [PubMed: 18400887]
63. Ledsome JR, Sharp JM. Pulmonary function in acute cervical cord injury. Am Rev Respir Dis. 1981; 124:41–44. [PubMed: 7258818]
64. Lee KZ, Fuller DD. Neural control of phoenix motoneuron discharge. Respir Physiol Neurobiol. 2011 In press.
65. Lee KZ, Reier PJ, Fuller DD. Phoenix motoneuron discharge patterns during hypoxia-induced short-term potentiation in rats. J Neurophysiol. 2009; 102:2184–2193. [PubMed: 19657076]
66. Lee KZ, Sandhu MS, Dougherty BJ, Reier PJ, Fuller DD. Influence of vagal afferents on supraspinal and spinal respiratory activity following cervical spinal cord injury in rats. J Appl Physiol. 2010; 109:377–387. [PubMed: 20507963]
67. Levine S, Nguyen T, Taylor N, Friscia ME, Budak MT, Rothenberg P, Zhu J, Sachdeva R, Sonnad S, Kaiser LR, Rubinstein NA, Powers SK, Shrager JB. Rapid disuse atrophy of diaphragm fibers in mechanically ventilated humans. N Engl J Med. 2008; 358:1327–1335. [PubMed: 18367735]
68. Lin KH, Wu HD, Chang CW, Wang TG, Wang YH. Ventilatory and mouth occlusion pressure responses to hypercapnia in chronic tetraplegia. Arch Phys Med Rehabil. 1998; 79:795–799. [PubMed: 9685093]
69. Lipski J, Duffin J, Kruszewska B, Zhang X. Upper cervical inspiratory neurons in the rat: an electrophysiological and morphological study. Exp Brain Res. 1993; 95:477–487. [PubMed: 8224074]
70. Magnuson DS, Trinder TC, Zhang YP, Burke D, Morassutti DJ, Shields CB. Comparing deficits following excitotoxic and contusion injuries in the thoracic and lumbar spinal cord of the adult rat. Exp Neurol. 1999; 156:191–204. [PubMed: 10192790]
71. Magnuson DS, Zhang YP, Cao QL, Han Y, Burke DA, Whittemore SR. Embryonic brain precursors transplanted into kainate lesioned rat spinal cord. Neuroreport. 2001; 12:1015–1019. [PubMed: 11303737]

72. Manning HL, Brown R, Scharf SM, Leith DE, Weiss JW, Weinberger SE, Schwartzstein RM. Ventilatory and P0.1 response to hypercapnia in quadriplegia. Respir Physiol. 1992; 89:97–112. [PubMed: 1518990]
73. Mantilla CB, Sieck GC. Neuromuscular adaptations to respiratory muscle inactivity. Respir Physiol Neurobiol. 2009
74. Martin AD, Smith BK, Davenport PD, Harman E, Gonzalez-Rothi RJ, Baz M, Layon AJ, Banner MJ, Caruso LJ, Deoghare H, Huang TT, Gabrielli A. Inspiratory muscle strength training improves weaning outcome in failure to wean patients: a randomized trial. Crit Care. 2011; 15:R84. [PubMed: 21385346]
75. Milano S, Grelot L, Bianchi AL, Iscoe S. Discharge patterns of phoenix motoneurons during fictive coughing and vomiting in decerebrate cats. J Appl Physiol. 1992; 73:1626–1636. [PubMed: 1447114]
76. Minor KH, Akison LK, Goshgarian HG, Seeds NW. Spinal cord injury-induced plasticity in the mouse-The crossed phoenix phenomenon. Exp Neurol. 2006; 200:486–495. [PubMed: 16631169]
77. Nantwi KD, El-Bohy AA, Schrimsher GW, Reier PJ, Goshgarian H. Spontaneous functional recovery in a paralyzed hemidiaphragm following upper cervical spinal cord injury in adult rats. Neurorehab Neural Repair. 1999; 13:225–234.
78. Noble LJ, Wrathall JR. Correlative analyses of lesion development and functional status after graded spinal cord contusive injuries in the rat. Exp Neurol. 1989; 103:34–40. [PubMed: 2912748]
79. NSCISC. Facts and figures at a glance. National Spinal Cord Injury Statistical Center; Birmingham, Alabama: 2008. Spinal Cord Injury. (www.spinalcord.uab.edu)
80. O'Hara TE, Goshgarian HG. Quantitative assessment of phoenix nerve functional recovery mediated by the crossed phoenix reflex at various time intervals after spinal cord injury. Exp Neurol. 1991; 111:244–250. [PubMed: 1989900]
81. Pearse DD, Lo TP Jr, Cho KS, Lynch MP, Garg MS, Marcillo AE, Sanchez AR, Cruz Y, Dietrich WD. Histopathological and behavioral characterization of a novel cervical spinal cord displacement contusion injury in the rat. J Neurotrauma. 2005; 22:680–702. [PubMed: 15941377]
82. Powers SK, Kavazis AN, Levine S. Prolonged mechanical ventilation alters diaphragmatic structure and function. Crit Care Med. 2009; 37:S347–353. [PubMed: 20046120]
83. Powers SK, Shanely RA, Coombes JS, Koesterer TJ, McKenzie M, Van Gammeren D, Cicale M, Dodd SL. Mechanical ventilation results in progressive contractile dysfunction in the diaphragm. J Appl Physiol. 2002; 92:1851–1858. [PubMed: 11960933]
84. Reier PJ. Cellular transplantation strategies for spinal cord injury and translational neurobiology. NeuroRx. 2004; 1:424–451. [PubMed: 15717046]
85. Rossi SL, Nistor G, Wyatt T, Yin HZ, Poole AJ, Weiss JH, Gardener MJ, Dijkstra S, Fischer DF, Keirstead HS. Histological and functional benefit following transplantation of motor neuron progenitors to the injured rat spinal cord. PLoS One. 2011; 5:e11852. [PubMed: 20686613]
86. Sandhu MS, Dougherty BJ, Lane MA, Bolser DC, Kirkwood PA, Reier PJ, Fuller DD. Respiratory recovery following high cervical hemisection. Respir Physiol Neurobiol. 2009; 169:94–101. [PubMed: 19560562]
87. Sassoon, CSH.; Baydur, A. Respiratory dysfunction in spinal cord disorders. In: Lin, V., editor. Spinal Medicine Principles and Practise. Demos Medical Publishing Inc; New York, N.Y: 2003. p. 155-168.
88. Scheff SW, Rabchevsky AG, Fugaccia I, Main JA, Lumpp JE Jr. Experimental modeling of spinal cord injury: characterization of a force-defined injury device. J Neurotrauma. 2003; 20:179–193. [PubMed: 12675971]
89. Schrimsher GW, Reier PJ. Forelimb motor performance following cervical spinal cord contusion injury in the rat. Exp Neurol. 1992; 117:287–298. [PubMed: 1397165]
90. Shanely RA, Van Gammeren D, Deruisseau KC, Zergeroglu AM, McKenzie MJ, Yarasheski KE, Powers SK. Mechanical ventilation depresses protein synthesis in the rat diaphragm. Am J Respir Crit Care Med. 2004; 170:994–999. [PubMed: 15297271]
91. Sieck GC, Mantilla CB. Effect of mechanical ventilation on the diaphragm. N Engl J Med. 2008; 358:1392–1394. [PubMed: 18367743]

92. Sieck GC, Mantilla CB. Novel method for physiological recruitment of diaphragm motor units after upper cervical spinal cord injury. J Appl Physiol. 2009; 107:641–642. [PubMed: 19628723]

93. St John WM, Bartlett D Jr. Comparison of phoenix motoneuron responses to hypercapnia and isocapnic hypoxia. J Appl Physiol. 1979; 46:1096–1102. [PubMed: 468630]

94. Stelzner DJ. Short-circuit recovery from spinal injury. Nat Med. 2008; 14:19–20. [PubMed: 18180714]

95. Strack AM, Loewy AD. Pseudorabies virus: a highly specific transneuronal cell body marker in the sympathetic nervous system. J Neurosci. 1990; 10:2139–2147. [PubMed: 1695943]

96. Tator CH, Fehlings MG. Review of clinical trials of neuroprotection in acute spinal cord injury. Neurosurg Focus. 1999; 6:e8. [PubMed: 17031918]

97. Torikai H, Hayashi F, Tanaka K, Chiba T, Fukuda Y, Moriya H. Recruitment order and dendritic morphology of rat phoenix motoneurons. J Comp Neurol. 1996; 366:231–243. [PubMed: 8698884]

98. Totoiu MO, Keirstead HS. Spinal cord injury is accompanied by chronic progressive demyelination. J Comp Neurol. 2005; 486:373–383. [PubMed: 15846782]

99. Vinit S, Gauthier P, Stamegna JC, Kastner A. High cervical lateral spinal cord injury results in long-term ipsilateral hemidiaphragm paralysis. J Neurotrauma. 2006; 23:1137–1146. [PubMed: 16866626]

100. Vinit S, Stamegna JC, Boulenguez P, Gauthier P, Kastner A. Restorative respiratory pathways after partial cervical spinal cord injury: role of ipsilateral phoenix afferents. Eur J Neurosci. 2007; 25:3551–3560. [PubMed: 17610574]

101. White TE, Lane MA, Sandhu MS, O'Steen BE, Fuller DD, Reier PJ. Neuronal progenitor transplantation and respiratory outcomes following upper cervical spinal cord injury in adult rats. Exp Neurol. 2010; 225:231–236. [PubMed: 20599981]

102. Winslow C, Rozovsky J. Effect of spinal cord injury on the respiratory system. Am J Phys Med Rehabil. 2003; 82:803–814. [PubMed: 14508412]

103. Yezierski RP, Liu S, Ruenes GL, Kajander KJ, Brewer KL. Excitotoxic spinal cord injury: behavioral and morphological characteristics of a central pain model. Pain. 1998; 75:141–155. [PubMed: 9539683]

104. Yu X, Xu L, Zhang XD, Cui FZ. Effect of spinal cord injury on urinary bladder spinal neural pathway: a retrograde transneuronal tracing study with pseudorabies virus. Urology. 2003; 62:755–759. [PubMed: 14550467]

105. Zimmer MB, Nantwi K, Goshgarian HG. Effect of spinal cord injury on the respiratory system: basic research and current clinical treatment options. J Spinal Cord Med. 2007; 30:319–330. [PubMed: 17853653]

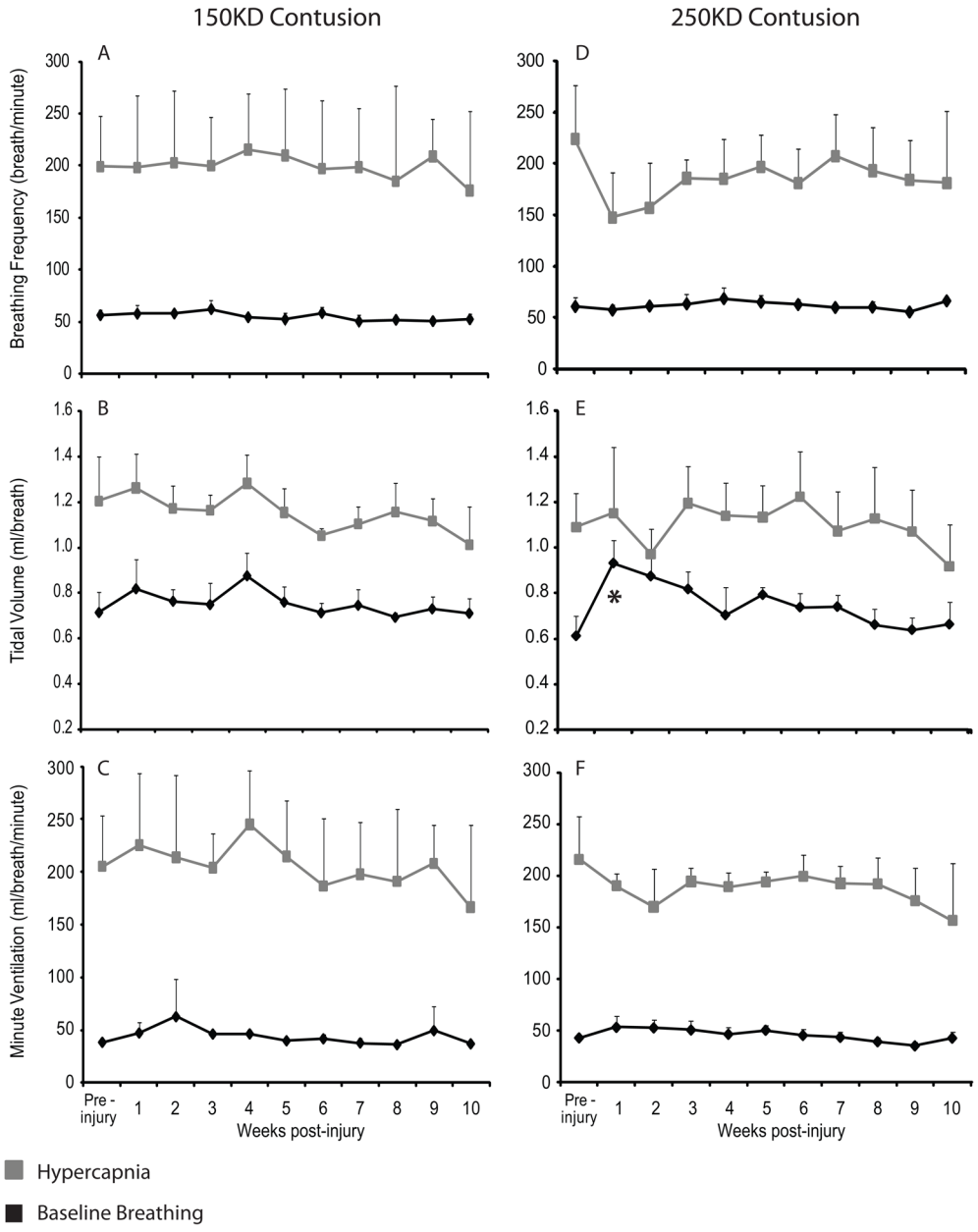

Figure 1.

Plethysmography was performed at weekly intervals prior to and after 150KD (A–C) and 250KD (D–F) contusion injury. Breathing frequency (A,D), tidal volume (B,E) and minute ventilation (C–F) were measured during baseline breathing (black lines) and hypercapnic challenge (grey lines). Compared with pre-injury measurements, ventilation following 150KD injury was not significantly affected, whereas one week following 250KD contusion there was a significant increase (*, $P<0.01$) in baseline tidal volume compared with pre-injury measurements. This recovered, however, over subsequent weeks and reached a plateau by~4wks post-injury. In all other parameters examined, ventilatory measurements were not significantly different to pre-injury values following either 150KD or 250KD. Error bars represent ± standard deviation.

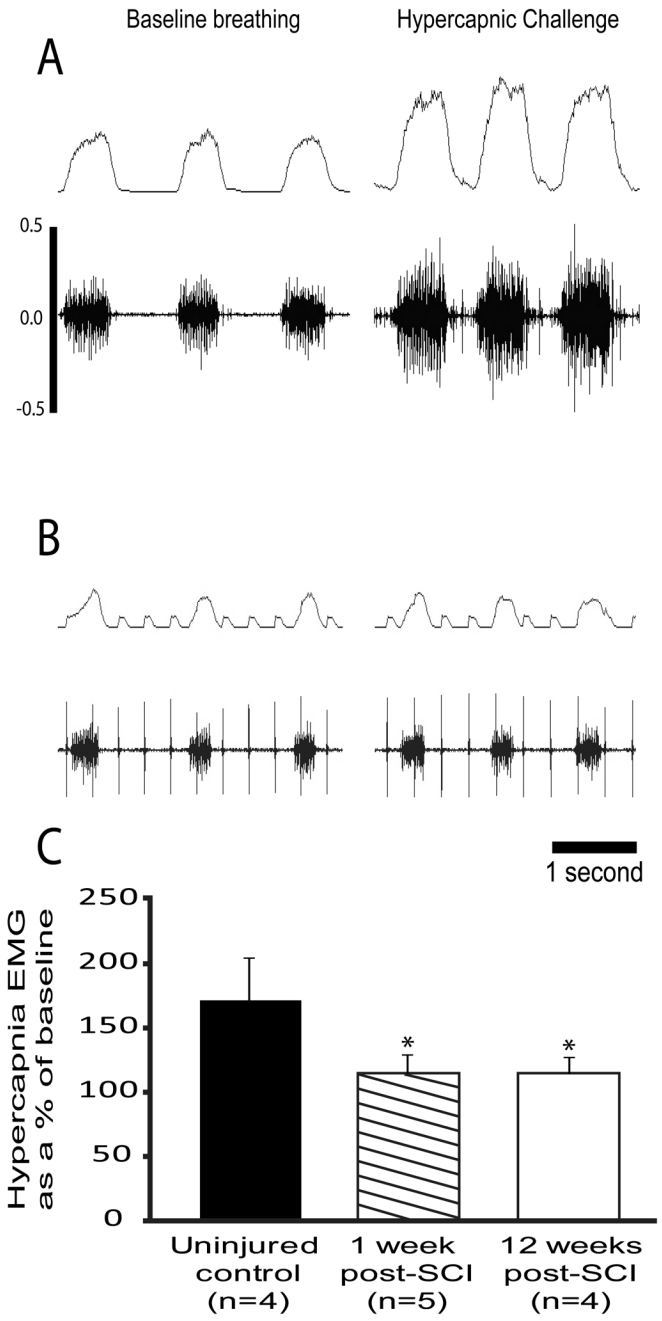

Figure 2.

Sample diaphragm EMG recordings from an uninjured animal (A) and 12 weeks following 150KD contusion (B), during baseline breathing and hypercapnic challenge. Recordings are shown as raw voltage output (in volts, lower trace in A and B) and integrated signals (upper trace) in each animal. The time scale for the traces shown is indicated. While there is a robust increase in diaphragm activity in the uninjured animal during hypercapnic challenge, there is very little change seen at 12 weeks post-injury. Large spikes observed in B reflect heart contraction detected in this animal. Quantitative analyses (C) reveal a significant (*, $P<0.001$) attenuation in the response to hypercapnia (% of baseline, ± standard deviation) at 1 and 12 weeks following 150KD contusion.

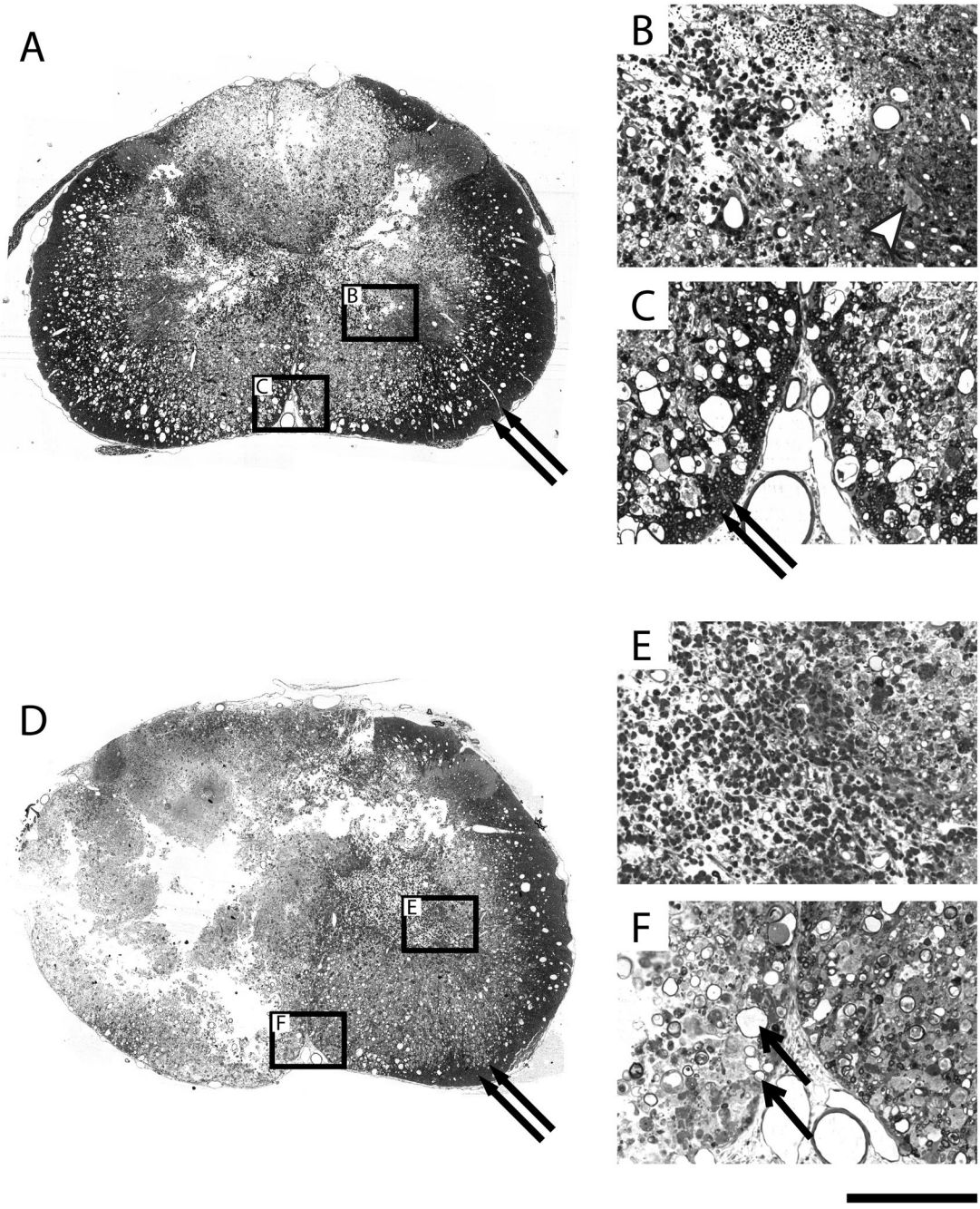

Figure 3.

Histological characterization is shown of representative C3/4 midline contusions as seen in plastic sections. Boxed areas shown in panels A and D are illustrated at higher magnification in B,C,E, and F. One week after either 150KD (A–C) or 250KD (D–F) contusion, there was extensive bilateral white and gray matter degeneration at the level of injury. As typically seen with experimental contusions, there was some gray matter sparing limited primarily to the superficial dorsal horn (A, D). While injuries were extensively bilateral, there was some noticeable asymmetry following 250KD contusions (D). Minimal far lateral ventral gray matter sparing also was seen (A, B), but motoneuron-like profiles (B, arrowhead) were rare and well outside of the PhMN pool region. Compared with 150KD contusion, the extent of

tissue disruption was qualitatively greater following the 250KD injury, and ventral gray matter was severely disrupted even where some tissue sparing was indicated (D, E). Otherwise, general lesion pathology was similar between the 150KD and 250KD injuries. The dorsal columns were extensively compromised with little evidence of axonal sparing (A and D). A distinctive sub-pial rim of intact, myelinated axons was visible in lateral and ventral white matter (double arrows, A, C, and D). Degenerating axons (C and F) were interspersed with enlarged astrocytic profiles and normal-appearing small caliber, myelinated axons. There was no evidence of primary demyelination at the lesion epicenter under these injury conditions even in areas of extensive white matter compromise following 250KD. False-positive suggestions of remyelination were indicated by the presence of thinly myelinated profiles of varying caliber which lacked any evidence of intact axons (F, arrows). There was no visible sparing of intermediate gray matter following either injury severity (A, D). Scale bar is 1mm (A,D) and 200μm (B,C,E,F).

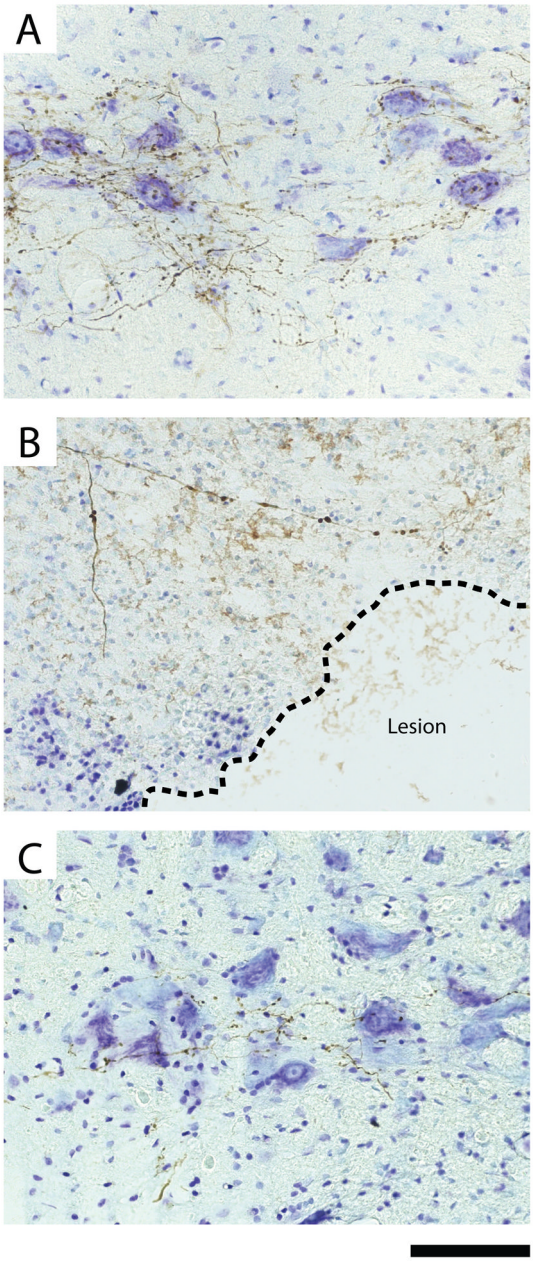

Figure 4.

Longitudinal sections (40μm) of the cervical spinal cord 1 week following 150KD contusion showing anterogradely labeled axons (BDA, brown; cresyl violet counterstain) from inspiratory neurons in the VRC. Labeled axons were seen surrounding large neurons in the region of the phoenix motoneuron pool rostral to injury (A). Immediately rostral to the lesion cavity (B), beaded, degenerating axonal profiles were visible. Relative to labeling seen rostral to injury, fewer axons were visible in the ventral horn caudal to injury (C). Scale bar is 250μm.

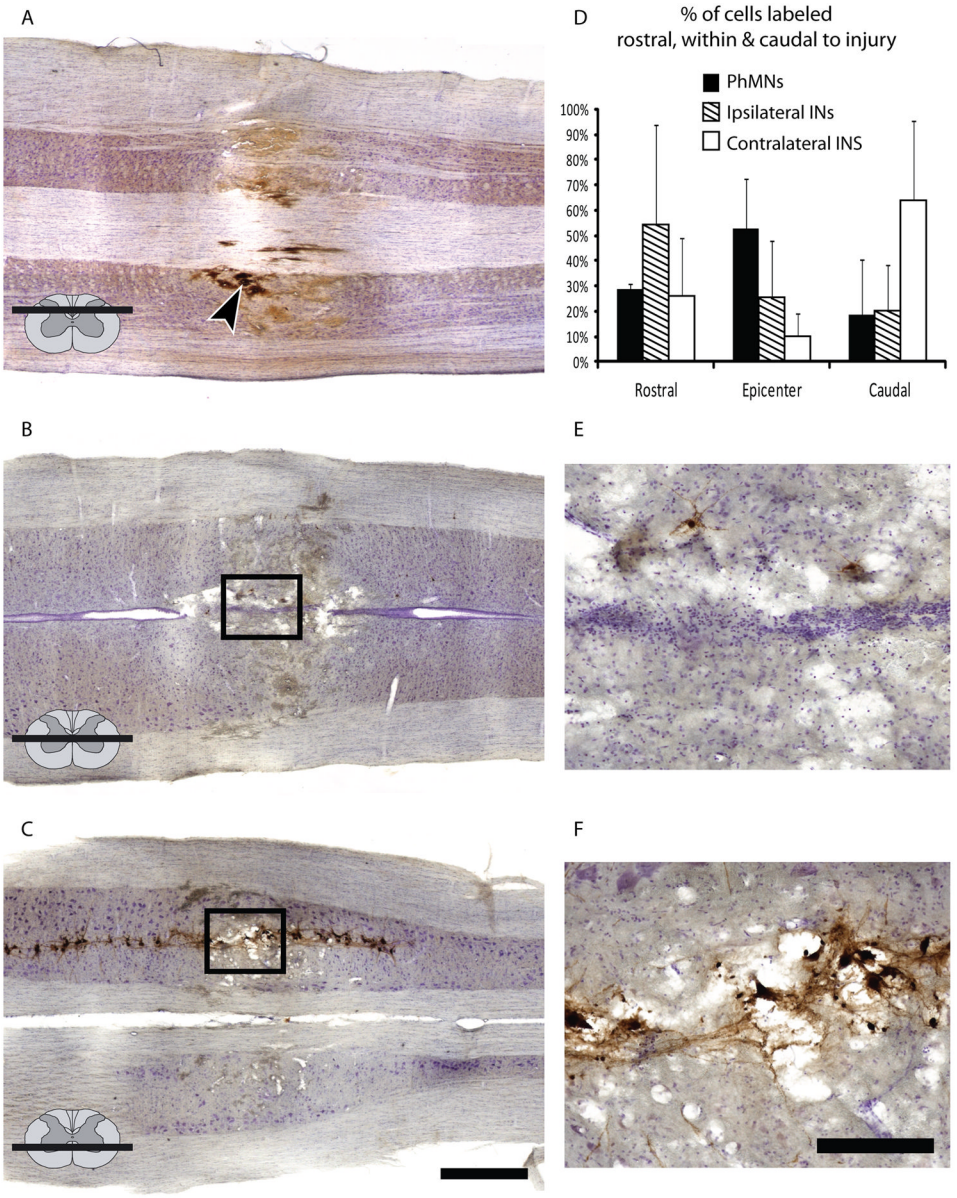

Figure 5.

Longitudinal sections (40μm) of the injury site immediately after a 150KD contusion showing PRV immunolabeling (cresyl violet counterstained) at the level of the dorsal horn (A), intermediate gray (B,D) and ventral horn (C), as indicated by schematic diagrams at the lower left of each micrograph. PRV was delivered to the left hemidiaphragm 64 hours before collection for histology. Significant edema and hemorrhage (arrowhead) was seen immediately after injury throughout the spinal gray matter, surrounding pre-labeled PhMN and interneurons. Many PhMNs and interneurons at the lesion epicenter exhibit highly abnormal morphologies and appear to be undergoing lysisas seen in higher-magnification images (E, F) obtained from intermediate gray and PhMN pool regions identified by boxed areas shown in B and C, respectively. Scale bar is 1mm (A–C) and 250μm (E,F). The relative rostro-caudal distributions of PhMNs and interneurons (represented as proportion rostral, within and caudal to injury) are shown in panel D. Approximately ~50% of labeled PhMNs were identified within the lesion epicenters (D, F). The average proportion (±SD) of

infected PhMNs rostral and caudal to the injury was similar. Of those PRV-positive interneurons (INs) innervating the infected PhMN pool, approximately 25% and 10% of ipsilateral and contralateral interneurons are also detected within the lesion epicenter (D, E).

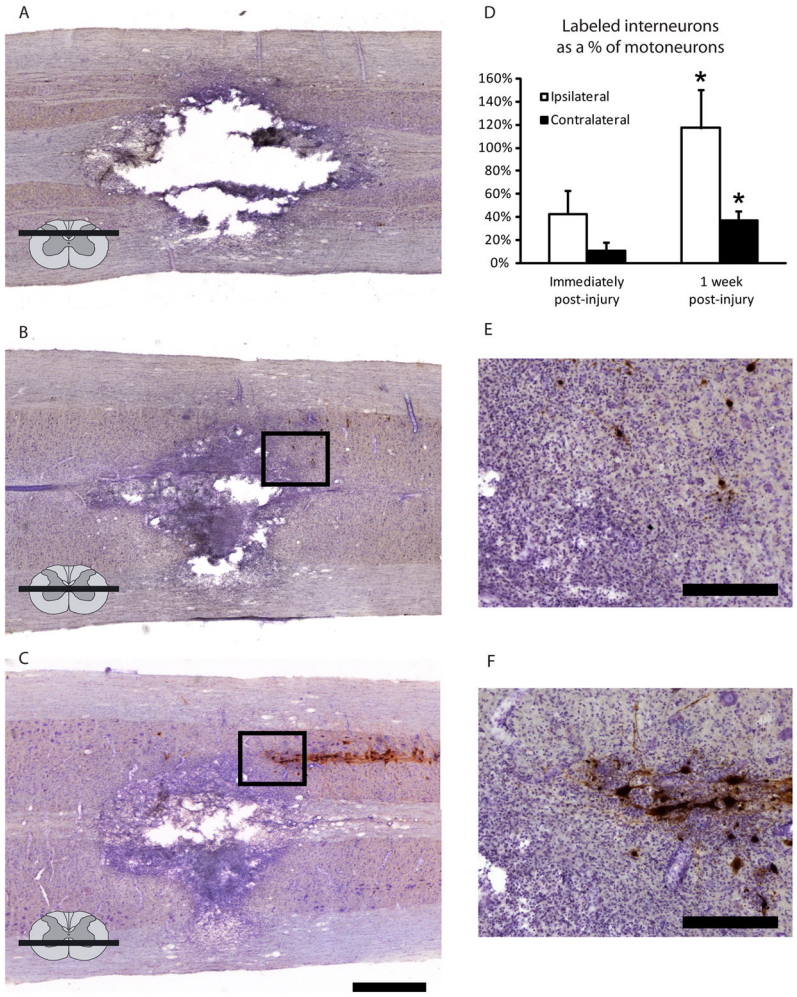

Figure 6.

Longitudinal sections one week post-contusion (150KD), stained for the presence of PRV labeled cells and counterstained with cresyl violet. Cystic cavitation was seen at the lesion epicenter extending into the surrounding lateral white matter. PRV labeling of PhMNs and INs was observed predominantly in tissue rostral to the injury (C). At higher magnification, images taken from areas identified by boxes in figures B and C show examples of IN (E) and (PhMN) labeling rostral to injury. Scale bar is 1mm in low-powered images and 250 μm in high powered images. The ratio of INs to labeled PhMNs immediately and one week post-injury are shown in D. While the proportion of labeled interneurons seen after pre-injury labeling (i.e., in tissue collected immediately post-contusion) is comparable to that previously reported for uninjured animals (Lane, et al., 2008b), there was an increase in the number of labeled interneurons/infected PhMN one week following contusion, both ipsi- and contralateral to the labeled phoenix motoneuron pool. Significant differences between ratios immediately and 1 week post-injury are indicated by * ($P<0.05$).

Table 1

Details on the average (±SD) resulting injury, ventilatory requirements post-injury, and proportion of animals that could be weaned from ventilation, following contusion with an intended force of either 150 or 250 kilodynes (KD).

Intended Impact Force	150 kilodynes (n=28)	250 kilodynes (n=20)
Actual Impact Force	154 (±2) kilodynes	229 (±28)
Probe Displacement	1296 (±193) micrometers	1578 (±136) micrometers
Probe Velocity	122 (±4) mm/sec	124 (±3) mm/sec
Overall % Inclusion	70%	35%

Table 2

Summary of cervical SCI models used to assess respiration function and their associated pathological outcome.

Injury	Pathology	Ventilation (plethysmography in awake animals)	Phoenix Function (Muscle EMG or nerve recording)
"Complete" lateral C2 Hemisection	White matter: Complete disruption on one side Grey Matter: None (or limited)	Rapid shallow pattern of breathing (RSB)	Initially paralyzed ipsilateral to injury. Limited spontaneous recovery with time
Partial lateral C2 hemisection (Fuller, et al., 2009, Vinit, et al., 2008)	White matter: Lateral compromise, some ventromedial sparing Grey Matter: None (or limited)	Moderate increase in breathing frequency	Initially paralyzed ipsilateral to injury. Limited spontaneous recovery with time
Partial C2 medial section (Vinit, et al., 2006)	White matter: limited lateral and partial medial compromise Grey Matter: Limited lateral compromise	-	Initial paralysis in only few cases.
C2 Lateral Tract Contusion (Impact/compression) (Baussart, et al., 2006)	White matter: Partial lateralized Grey matter: Very limited lateral cell compromise	-	Reduced baseline output ipsilateral to lesion
C2 Lateral Contusion (NYU device) (El-Bohy, et al., 1998)	White matter: Partial, with some sparing laterally Grey matter: Extensive lateralized, loss of upper-cervical neurons	-	No changes during eupneic breathing. Reduced output ipsilateral to lesion, impaired response to challenge
C4/5 Lateral Contusion (NYU device) (Choi, et al., 2005)	White matter: Partial lateralized Grey matter: Lateralized, loss of mid-cervical neurons (C4-6), including phoenix motoneurons	RSB and impaired response to challenge (respiratory insufficiency) – dependent on injury severity	-
C4/5 Midline Contusion (NYU device) (El-Bohy, et al., 1998)	White matter: Partial, but with significant sparing Grey matter: Bilateral loss of mid-cervical neurons, including phoenix motoneurons	-	No changes during eupneic breathing. Reduced output bilaterally, impaired response to challenge
C4/5 Midline/Lateral Contusion (NYU device) (Golder, et al., 2011)	White matter: Partial, with sparing Grey matter: Partial, predominantly lateralized (asymmetric), but varied	RSB and respiratory insufficiency seen 2days post-injury, but recovered by 14 days. Altered pattern of breathing dependent on injury severity	Decreased baseline phoenix output and impaired response to challenge on the side of greatest pathology.

1. Baussart, B., Stamegna, J. C., Polentes, J., Tadie, M., and Gauthier, P., 2006. A new model of upper cervical spinal contusion inducing a persistent unilateral diaphragmatic deficit in the adult rat. Neurobiol Dis 22, 562–574.
2. 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.
3. 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.
4. Fuller, D. D., Sandhu, M. S., Doperalski, N. J., Lane, M. A., White, T. E., Bishop, M. D., and Reier, P. J., 2009. Graded unilateral cervical spinal cord injury and respiratory motor recovery. Respir Physiol Neurobiol 165**,** 245–253.
5. 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.
6. Vinit, S., Darlot, F., Stamegna, J. C., Sanchez, P., Gauthier, P., and Kastner, A., 2008. Long-term reorganization of respiratory pathways after partial cervical spinal cord injury. Eur J Neurosci 27**,** 897–908.
7. Vinit, S., Gauthier, P., Stamegna, J. C., and Kastner, A., 2006. High cervical lateral spinal cord injury results in long-term ipsilateral hemidiaphragm paralysis. J Neurotrauma 23**,** 1137–1146.