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TITLE: **Improving Survival and Promoting Respiratory Motor Function After Cervical Spinal Cord Injury**

PRINCIPAL INVESTIGATOR: **Warren J. Alilain, Ph.D.**

CONTRACTING ORGANIZATION: **UNIVERSITY OF KENTUCKY**
500 S LIMESTONE 109 KINKEAD HALL
LEXINGTON KY 40526-0001

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14. ABSTRACT Most spinal cord injuries (SCI) occur near the neck. The cervical level of the spinal cord is located near this area. At this level are the connections to the cells which instruct the diaphragm, the major muscle used for breathing, to contract. Therefore, damage at the cervical level can lead to a paralyzed diaphragm and an inability to breathe and inspire air. Patients who survive this type of injury often need the use of a mechanical ventilator in order to survive. Use of the ventilator severely limits the quality of life of those injured and dramatically increases the demand for health care. However, despite these drastic interventions, the cervical injured patient is still susceptible to death due to respiratory complications. This application proposes to help improve survival, decrease early dependence on mechanical ventilation, and restore breathing after cervical spinal cord injury, as well as develop prognostic indicators for injury progression and recovery. Through these studies we endeavor to add to the standard of care for cervical SCI patients so as to lead to an improved quality of life, better-quality health care management, and improved functional outcomes.					
15. SUBJECT TERMS SCI, breathing, phrenic nucleus, regeneration, neurotrauma, variability, pattern					
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1. Introduction

More than 50% of spinal cord injuries (SCI) occur near the neck. The cervical level of the spinal cord is located near this area. At the cervical level are the connections to the neurons which induces the diaphragm, the major muscle used for breathing, to contract. Therefore, damage at the cervical level can lead to a paralyzed diaphragm and an inability to breathe and inspire air. Patients who survive this type of injury often need the use of a mechanical ventilator in order to survive. Use of the ventilator severely limits the quality of life of those injured and dramatically increases the demand for health care. However, despite these drastic interventions, the cervical injured patient is still susceptible to death due to respiratory complications, including atelectasis, pneumonia, and acute respiratory distress syndrome. This project proposes to help improve survival, decrease early dependence on mechanical ventilation, and restore breathing after cervical spinal cord injury, as well as develop prognostic indicators for injury progression and recovery. Through these studies we endeavor to add to the standard of care for cervical SCI patients so as to lead to an improved quality of life, better-quality health care management, and improved functional outcomes of this severely debilitated population.

2. Keywords

SCI, breathing, phrenic nucleus, axon, regeneration, neurotrauma, variability, pattern

3. Accomplishments

What are the major goals of the project?

The major goals of the project are to:

- 1) Obtain regulatory review and approval by USAMRMC Animal Care and Use Review Office (ACURO) and local Institutional Animal Care and Use Committee (IACUC).
- 2) Test the hypothesis that inhibiting PTEN through bpV will promote recovery of respiratory motor function after cervical SC contusion.
- 3) Test the hypothesis that increasing respiratory drive immediately after cervical contusion will promote survival and independence immediately after cervical SCI.
- 4) Test the hypothesis that respiratory motor patterns and variability are surrogate endpoints that accurately predict functional improvement after cervical SC contusion and intervention.

Task/Milestone	Target Completion Date/Quarter	Status
Major Goal 1	Y1 Q1	completed, 100%
Major Goal 2	Y2 Q4	in progress, 75%
Major Goal 3	Y3, Q4	in progress, 40%
Major Goal 4	Y3, Q4	in progress, 20%

What was accomplished under these goals?

As the Science Officers, Drs. Quntian Wang and Jessica Tilghman, and Grants Specialist, Amber Stillrich, are aware, in the first year of the award I changed institutions and am now located in

the Spinal Cord and Brain Injury Research Center at the University of Kentucky (UK) in Lexington, KY. This move was effective 10/1/15.

However, with this move there was a delay in obtaining approval of an award transfer to the University of Kentucky. This was accomplished 8/16/16.

In effect, the first year of the award was in reality 1.5 months of actual time devoted to the project (8/15/15 (project start date)-10/1/15(day I transferred UK)).

FIRST YEAR

Major Goal 1: Obtain regulatory review and approval by USAMRMC Animal Care and Use Review Office (ACURO) and local Institutional Animal Care and Use Committee (IACUC).

We accomplished the first major goal of the project which was obtaining regulatory review and approval by the USAMRMC Animal Care and Use Review Office (ACURO) and the local Institutional Animal Care and Use Committee (IACUC) at the University of Kentucky. IACUC approval was obtained 2/22/16. ACURO approval was later attained 3/24/16. This was accomplished before official transfer of the award to the University of Kentucky.

SECOND YEAR

Major Goal 2: Test the hypothesis that inhibiting PTEN through bpV will promote recovery of respiratory motor function after cervical SC contusion.

In the second year of the award, newly hired staff and incoming students were trained in the proper methods and approaches of performing cervical SCI's, which is a key component of this project. Much time was devoted to this training as cervical SCI is a complicated and difficult surgery and much practice is needed in order to produce consistent injury outcomes. Additionally, staff and students were trained to perform diaphragmatic EMG recordings, which will be used to measure key functional outcomes after injury and treatment. These project specific techniques were in addition to the acquisition of general animal handling skills, including post surgery care and drug administration.

After this long period of training, we initiated experiments as outlined in the proposal. Specifically, evaluation of the therapeutic agent, bisperoxovanadium (bpV), a potent PTEN inhibitor, to promote rescue of damaged bulbospinal pathways which mediates breathing. Results thus far have been encouraging. Alongside evaluating bpV as the lone treatment, we combined bpV with rehabilitative training in the form of repeated intermittent hypoxia (IH) exposures. This combined therapeutic approach appears to have yielded the best results in promoting the rescue of damaged respiratory pathways and breathing activity. Control treated animals (no bpV or IH) or IH alone did not yield repair of damaged pathways and promotion of breathing function.

THIRD YEAR

Major Goal 2: Test the hypothesis that inhibiting PTEN through bpV will promote recovery of respiratory motor function after cervical SC contusion.

We completed quantification and analysis of the role inhibition of PTEN through systemic bpV administration has on on respiratory motor function and spinal pathways following acute cervical spinal cord injury (SCI). This involved performing hemicontusions and administration of pharmacological agents (saline or bpV) with or without post-injury rehabilitation (normoxia or IH).

Experiments determined that a combination treatment (bpV/IH) promotes significant effects on breathing activity time and amplitude per breath following cervical SCI. In short, animals treated with this combination therapy can effectively utilize damaged pathways to breathe. The remaining treatments (saline/normoxia) and (saline/IH) were not significant. While experiments indicated that bpV alone might have been helpful in promoting activity follow cervical SCI this was determined to not be of significance (although it was the second most effective treatment).

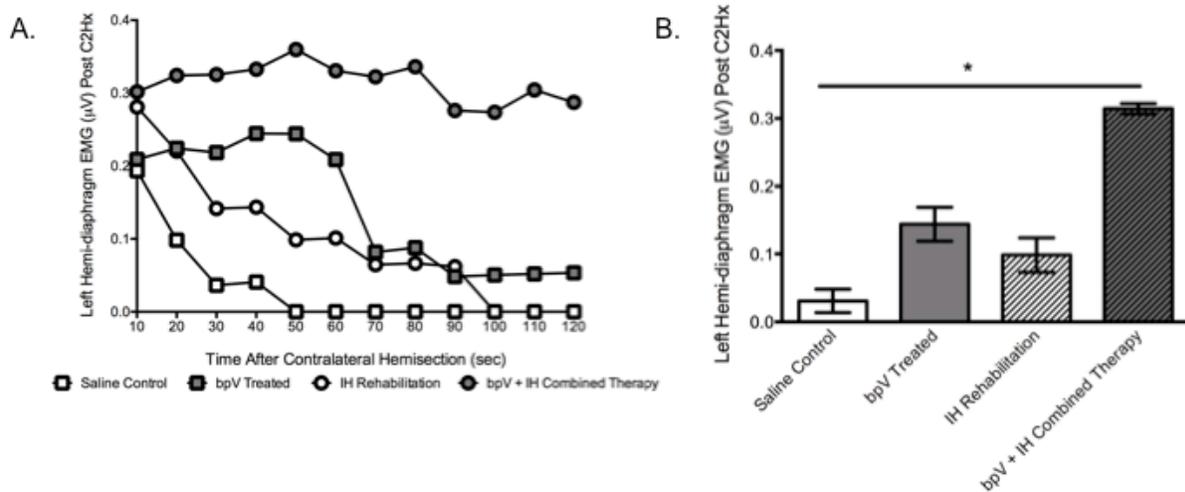


Figure 1. bpV treatment and IH rehabilitation enhances activity of damaged respiratory motor pathways. Following a left unilateral cervical contusion injury, animals were treated with combinations of saline or bpV, with or without IH rehabilitation. Following right contralateral C2 hemisection to isolate diaphragmatic activity solely to contused pathways, the combination of bpV and IH helps rescue these pathways from damage and promotes activity of the ipsilateral diaphragm.

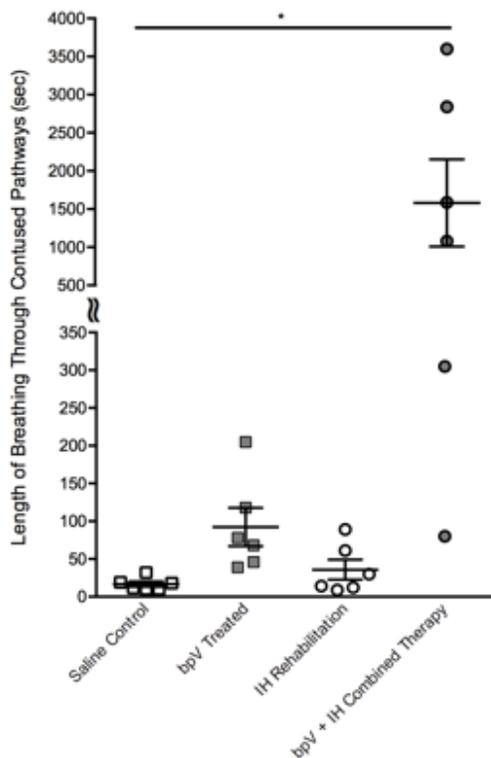


Figure 2. bpV treatment and IH rehabilitation rescues breathing activity of damaged respiratory motor pathways. Following a left unilateral cervical contusion injury, animals were treated with combinations of saline or bpV, with or without IH rehabilitation. Following right contralateral C2 hemisection to isolate diaphragmatic activity solely to contused pathways, animals cannot utilize damaged pathways to maintain diaphragmatic activity if untreated. However, if treated with bpV and IH, breathing activity is restored suggesting rescue of activity of these damaged pathways. Saline, bpV

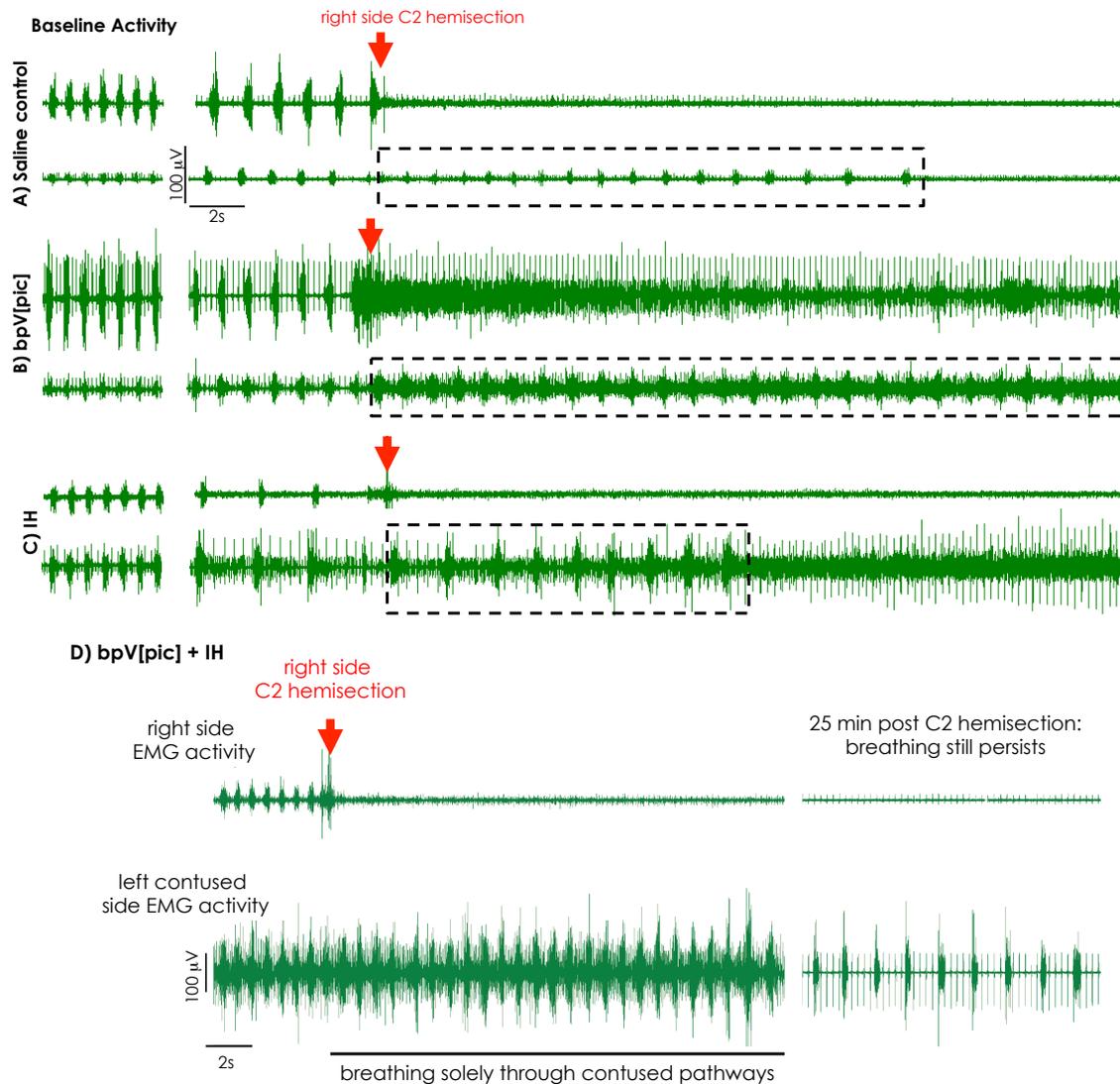


Figure 3. Representative traces of bilateral diaphragmatic activity of saline, bpV, IH, and bpV and IH treated animals. Cervical SC contused animals which received both bpV and IH exhibited the longest amount of diaphragmatic activity produced solely through damaged pathways compared to other groups. Red arrow indicates time when contralateral C2 hemisection performed to isolate breathing activity solely to damaged pathways.

Major Goal 3: Test the hypothesis that increasing respiratory drive immediately after cervical contusion will promote survival and independence immediately after cervical SCI. Our experiments have determined that pre-treatment with theophylline, a respiratory stimulant, that is approved for human use can promote survival following cervical contusion SCI.

Adult rats received either systemic intraperitoneal injections of theophylline (15 mg/kg) or saline vehicle treatment immediately before receiving a 150 kD lateral contusion injury. Following this severe injury **all** saline treated animals did not survive up to the 10 minute post-contusion time point. Conversely, theophylline treated animals survived at least to the 10 minute post contusion time point. Qualitatively, saline treated animals displayed reduced breathing frequency immediately following the contusion injury which did not resolve and ultimately led to a

cessation of breathing. Theophylline treated animals displayed robust breathing shortly after injury.

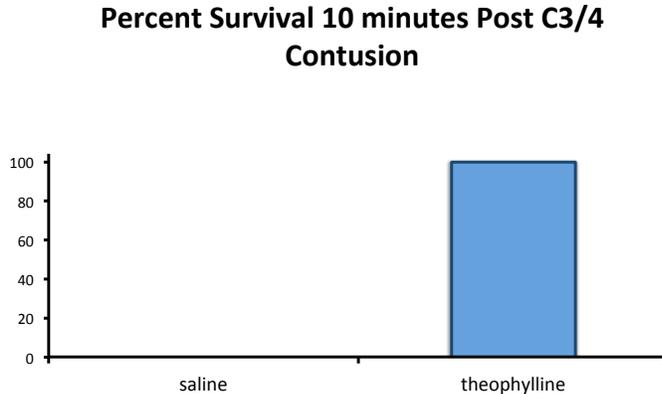


Figure 3. Theophylline pre-treatment leads to robust breathing and survival after cervical contusion spinal cord injury. Theophylline at 15 mg/kg administered systemically before injury led to robust breathing and long term survival (>10 min). Saline treated animals displayed weak breathing following cervical injury ultimately leading to a cessation of breathing.

Major Goal 4: Test the hypothesis that respiratory motor patterns and variability are surrogate endpoints that accurately predict functional improvement after cervical SC contusion and intervention.

Experiments have determined that following cervical contusion injury animals displayed significant functional deficits which lasted from acute to chronic time points. First, injured animals could not utilize isolated contused pathways to maintain respiratory activity. This was observed in multiple respiratory musculature. Secondly, whole body plethysmography indicated that injured animals displayed reductions in tidal and minute volumes. Lastly, injury led to a significant decrease in ventilatory pattern variability. These results were published in Warren et al., 2018.

These results set the foundation for Major Task 4. Our model of cervical contusion injury leads to robust and persistent deficits in muscle function and ventilation, as well as alterations in ventilatory pattern variability. As our results suggests that bpV treatment alongside IH rehabilitation can promote significant repair/rescue of contused pathways to promote breathing our next steps will be to determine if there is a return of pattern variability towards pre-injury levels indicating such repair.

Collectively, this year was a productive and exciting one. From our experiments we can conclude that respiratory motor pathways injured by contusion can be repaired and made more effective with PTEN inhibition in conjunction with task specific rehabilitation. Furthermore, by increasing respiratory drive we can promote survival following devastating cervical contusion injury. Lastly, we have developed the groundwork to utilize ventilatory pattern variability analysis as a tool for tracking and predicting injury and treatment outcomes. These findings have clear translational relevance to those who experience cervical spinal cord injury, including our servicemen and women.

What opportunities for training and professional development did the project provide?

Staff and graduate students at the Spinal Cord and Brain Injury Research Center were provided specialized training in how to perform cervical spinal cord injuries and the necessary post-surgical care.

How were the results disseminated to communities of interest?

Results were disseminated primarily through invited seminars given by the P.I., Dr. Alilain. Specifically for this reporting year, results of this work was presented at:

February 19, 2018

Department of Neuroscience, University of Kentucky, Lexington, KY

April 16, 2018

Intermittent Hypoxia Retreat, University of Florida, Gainesville, FL

July 27, 2018

Department of Physiology, University of Florida, Gainesville, FL

There was one publication:

Warren PM, Campanaro C, Jacono FJ, **Alilain WJ**. Mid-cervical spinal cord contusion causes robust deficits in respiratory parameters and pattern variability. *Exp Neurol*, 306: 122-131 (2018).

What do you plan to do during the next reporting period to accomplish the goals and objectives?

For Major Goal 2, we will submit for publication our findings on PTEN inhibition and IH exposure to promote respiratory motor recovery after cervical SCI. We are still investigating the anatomical substrate of recovery.

For Major Goal 3, we are encouraged by our initial results and will expand our experiments to determine the effect of theophylline treatment **after** cervical contusion injury which is more clinically relevant. Additionally, we propose to utilize more severe contusion injuries to determine the effectiveness of this therapy.

For Major Goal 4, we will continue our experiments and quantify ventilatory pattern variability after injury and treatment.

4. Impact

What was the impact on the development of the principal discipline(s) of the project?

Collectively, the impact of these findings is significant:

Our studies will be the first to suggest that damaged *respiratory* pathways can be made more efficacious to support breathing when left entirely on their own. We can make this statement since previous studies have utilized the lateral C2 hemisection model of SCI to study respiratory motor insufficiency, but in this model recovery is dependent upon the *uninjured* crossed phrenic pathway which circumvents the lesion. Other studies utilizing cervical contusion injuries do not isolate any recovery observed solely to injured pathways and therefore recovery can still be partially attributed to uninjured pathways. These studies can serve as the launching point to further examine the potential of bpV to inhibit PTEN and promote recovery in chronic SCI. Ultimately, if these studies indicate some beneficial effect of bpV administration, translation of these pre-clinical therapeutic approaches to the human SCI population can begin to be considered.

Secondly, our results demonstrate that survival after cervical SCI can be improved by increasing respiratory drive. This was accomplished through administration of theophylline. As

theophylline is an already approved for human use drug, translation to the human injury setting can be immediate. We envision theophylline treatment as a “first order” treatment option when cervical injuries are sustained. This can dramatically improve survival. Scientifically, it will be interesting to determine the exact mechanism behind recovery. This could lead to future studies exploiting these uncovered mechanisms to promote functional recovery.

Lastly, our use of ventilatory pattern variability analysis in the setting of spinal cord injury is the first instance. We are the first to demonstrate that pattern variability is reduced after SCI and this deficit is maintained up to chronic timepoints. Its use to determine future health outcomes after injury and treatment will be determined in the future.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report.

5. Changes/Problems

The only major change encountered so far has been the change in institution for the contract. This process took some time and was longer than anticipated leading to reduced time in the first year (essentially 1.5 months of actual project work).

Problems that were initially encountered include a high mortality rate acutely after injury. This could be partially attributed to the relative inexperience of the staff and students performing the surgeries and injuries.

In the last year a major obstacle encountered was a knee injury and subsequent surgery experience by the PI which resulted in some time away from the laboratory. Dr. Tilghman was made aware of this.

6. Products

As indicated above, products from the previous year included presentations and a publication:

February 19, 2018

Department of Neuroscience, University of Kentucky, Lexington, KY

April 16, 2018

Intermittent Hypoxia Retreat, University of Florida, Gainesville, FL

July 27, 2018

Department of Physiology, University of Florida, Gainesville, FL

Warren PM, Campanaro C, Jacono FJ, **Alilain WJ**. Mid-cervical spinal cord contusion causes robust deficits in respiratory parameters and pattern variability. *Exp Neurol*, 306: 122-131 (2018).

7. Participants & Other Collaborating Organizations

Warren J. Alilain, Ph.D.

Principal Investigator

Nearest person month worked: 3

Dr. Alilain is the Principal Investigator of this project and therefore keeps updated and knowledgeable of the relevant fields (SCI, respiratory plasticity, experimental interventions, etc.). He is also the primary author of the Animal Protocols submitted to ACURO and the University of Kentucky IACUC. Dr. Alilain has also trained all the personnel in the appropriate and necessary skills to conduct the experiments and complete the goals of this experiment. He directs experimental design, data collection and analysis. He presents findings through seminar presentations, as well as in the composition of manuscripts.

Frank J. Jacono, M.D.

Optional Qualified Collaborator

Nearest person month worked: 1

Dr. Jacono is the Optional Qualified Collaborator of this project and helps oversee the quantification, analysis, and interpretation of the various quantitative measures, as well as the ventilatory pattern variability evaluations. He assists in manuscript preparation.

Chris Calulot

Research Technician/Analyst

Nearest person month worked: 4

Mr. Calulot learned the necessary animal procedures necessary for the completion of this project. He participates in performing the cervical contusion injury.

Cara Campanaro

Research Technician/Analyst

Nearest person month worked: 6

Ms. Campanaro is a technician of Dr. Jacono. She analyzed ventilatory pattern variability after cervical SCI ranging from acute to chronic time points.

Philippa M. Warren, Ph.D.

Post-doc

Dr. Warren was a post-doc in the laboratory of Dr. Alilain. She performed injury surgeries and analyzed motor and plethysmography data. She is the first author of the publication describing the motor, ventilatory, and variability deficits resulting from contusion injury: Warren et al., 2018. Dr. Warren was supported by her own funding.

8. Special Reporting Requirements

please see attached Quad Chart in Appendix

9. Appendices – please see quad chart

Improving Survival and Promoting Respiratory Motor Function After Cervical Spinal Cord Injury

W81XWH-15-1-0378, SC140243

PI: Warren J. Alilain, Ph.D.



Study/Product Aim(s)

The major goals of the project are to:

- 1) Obtain regulatory review and approval by USAMRMC Animal Care and Use Review Office (ACURO) and local Institutional Animal Care and Use Committee (IACUC).
- 2) Test the hypothesis that inhibiting PTEN through bpV will promote recovery of respiratory motor function after cervical SC contusion.
- 3) Test the hypothesis that increasing respiratory drive immediately after cervical contusion will promote survival and independence immediately after cervical SCI.
- 4) Test the hypothesis that respiratory motor patterns and variability are surrogate endpoints that accurately predict functional improvement after cervical SC contusion and intervention.



Our work seeks to improve survival and restore breathing function after cervical spinal cord injury. Left image is of Matt Hampson, an individual with a cervical SCI, on a ventilator which helps enable him to breathe. We want to remove the need for mechanical ventilation. Photo from: www.spinal-research.org. <http://www.spinal-research.org/news/spinal-research-supporter-matt-hampson-speaks-about-spinal-cord-injury-on-channel-4%E2%80%99s-embarrassing-bodies/>

Schedule

Activities	16	17	18	19
1) Get IACUC and ACURO approval	■			
2) Inhibit PTEN after injury		■	■	
3) Enhance respiratory drive after injury		■	■	■
4) Record and evaluate respiratory motor patterns		■	■	■

Created: 11/29/2016; Modified: 9/28/2018

Goals/Milestones:

Milestone: Local IRB/IACUC Approval

Milestone: HRPO/ACURO Approval

Milestone 1: Respiratory motor outcomes following treatments will be quantified.

Milestone 2: Optimal bpV dosage for improved respiratory motor determined mediated by damaged pathways will be determined.

Milestone 3: The effects of bpV treatment on tissue sparing, sprouting of 5-HT fibers, and regeneration associated signaling molecules will be quantified.

Milestone 4: Respiratory motor outcomes following bpV and rehabilitation will be quantified.

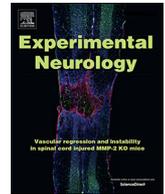
Milestone 5: The effects of bpV treatment and IH rehabilitation on SC tissue will be quantified.

Milestone 1: The effect of increasing respiratory drive with theophylline administration on improving survival and independence on mechanical ventilation immediately after cervical SCI will be quantified.

Milestone 2: Lesion volume will be characterized and quantified.

Milestone 1: Correlate patterns of altered ventilatory pattern variability with the severity of SCI, survival and response to pharmacological intervention (bpV and theophylline).

Black: completed Green: in progress



Research Paper

Mid-cervical spinal cord contusion causes robust deficits in respiratory parameters and pattern variability

Philippa M. Warren^{a,b}, Cara Campanaro^c, Frank J. Jacono^c, Warren J. Alilain^{a,d,*}

^a Department of Neurosciences, MetroHealth Medical Center, Case Western Reserve University, 10900 Euclid Avenue, Cleveland, OH 44106, USA

^b School of Biomedical Sciences, Faculty of Biological Sciences, University of Leeds, Leeds LS2 9JT, United Kingdom

^c Division of Pulmonary Critical Care and Sleep Medicine and Louis Stokes VA Medical Center, Department of Medicine, Case Western Reserve University, 10900 Euclid Avenue, Cleveland, OH 44106, USA

^d Spinal Cord and Brain Injury Research Centre, University of Kentucky, Lexington, KY 40536, USA



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Minute volume
Tidal volume
Respiratory EMG

ABSTRACT

Mid-cervical spinal cord contusion disrupts both the pathways and motoneurons vital to the activity of inspiratory muscles. The present study was designed to determine if a rat contusion model could result in a measurable deficit to both ventilatory and respiratory motor function under “normal” breathing conditions at acute to chronic stages post trauma. Through whole body plethysmography and electromyography we assessed respiratory output from three days to twelve weeks after a cervical level 3 (C3) contusion. Contused animals showed significant deficits in both tidal and minute volumes which were sustained from acute to chronic time points. We also examined the degree to which the contusion injury impacted ventilatory pattern variability through assessment of Mutual Information and Sample Entropy. Mid-cervical contusion significantly and robustly decreased the variability of ventilatory patterns. The enduring deficit to the respiratory motor system caused by contusion was further confirmed through electromyography recordings in multiple respiratory muscles. When isolated via a lesion, these contused pathways were insufficient to maintain respiratory activity at all time points post injury. Collectively these data illustrate that, counter to the prevailing literature, a profound and lasting ventilatory and respiratory motor deficit may be modelled and measured through multiple physiological assessments at all time points after cervical contusion injury.

1. Introduction

Inspiration in mammals is largely caused by the contraction of the diaphragm. This powerful muscle is innervated by the phrenic motor pool, located between the third and fifth cervical spinal cord segments (C3–C5) in humans and rodents (Ellenberger and Feldman, 1988; Keswani and Hollinshead, 1955; Mantilla et al., 2009). Additional, accessory muscles are involved in facilitating inspiration. This includes the external intercostals (innervated at thoracic level T1–T12) and the pharyngeal dilator muscles (such as the genioglossus) (Drake et al., 2015). The latter muscles are important modulators of upper airway patency and innervated through the hypoglossal nerve, not via any spinally mediated pathway. Inspiratory activity is elicited in the rostral ventral respiratory group located within the medulla (Duffin, 2004; Feldman et al., 1985). Due to damage of the respiratory motoneurons and bulbospinal pathways, cervical spinal cord injury (SCI) causes respiratory compromise experimentally and clinically (DeVivo and Chen, 2011). Indeed, respiratory dysfunction is the major cause of morbidity

and mortality following human cervical SCI (Brown et al., 2006; Hartkopp et al., 1997; Winslow and Rozovsky, 2003), with the mid-cervical contusion being the most prevalent form of trauma (Young, 2002).

Animal models of mid-cervical spinal cord contusion have explored the changes this injury evokes in respiratory function. Several studies have demonstrated a significant decrease in diaphragm electromyography (EMG), phrenic nerve activity, or ventilatory capacity under challenge (Awad et al., 2013; Baussart et al., 2006; el-Bohy et al., 1998; Golder et al., 2011; Lee et al., 2017; Lee and Kuo, 2017; Stamegna et al., 2011; Wen and Lee, 2017). For example, Wen and Lee (2017) have recently shown that tidal volume (VT) is reduced up to eight weeks following a cervical level 3/4 (C3/4) unilateral contusion. Interestingly, they show only transient effects upon inspiratory bursting in the bilateral diaphragm and external intercostal muscles, suggestive of some compensatory or endogenous plasticity occurring to facilitate function within this motor system. Indeed, a number of studies detail only a modest effect of mid-cervical contusion upon respiratory motor

* Corresponding author at: BBSRB B469, 741 S. Limestone Street, University of Kentucky, Lexington, KY 40536, USA.
E-mail address: warren.alilain@uky.edu (W.J. Alilain).

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parameters (Alvarez-Argote et al., 2016; Choi, 2005; Gensel et al., 2006; Lane et al., 2012; Nicaise et al., 2012). While animals demonstrate an initial inability to respond to hypoxic or hypercapnic challenge (Alvarez-Argote et al., 2016; Choi, 2005; Lane et al., 2012), they typically show recovery in all respiratory parameters by four-to-eight weeks post trauma. However, this does not reflect the clinical situation. Following mid-cervical contusion, some patients may be able to be weaned off ventilation (Bluehardt et al., 1992; Brown et al., 2006), however, respiratory function does not typically return to “normal”, pre-injury values (Winslow and Rozovsky, 2003). An important question is how to achieve a model of respiratory contusion which accurately mimics clinical outcome measures overtime, and whether we can deepen this analysis to provide further insight into the effect of injury on respiratory function?

Respiratory control is known to be complex and exhibit deterministic behaviour (Askanazi et al., 1979; Brack et al., 2002; Ibrahim et al., 2008). A number of disorders lead to a decrease in ventilatory pattern variance including respiratory failure (Wysocki et al., 2006), sepsis (Askanazi et al., 1979), and restricted lung disease (Brack et al., 2002). As mid-cervical contusion damages respiratory motor pathways, and reduces the number of motoneurons, we hypothesised that this injury would cause a decrease in variability of the waveform pattern. Indeed, utilising a series of recently developed analytical tools (Chung et al., 2013; Dhingra et al., 2011), we quantified deterministic variance within the ventilatory pattern of unilateral mid-cervically contused rats from three days to twelve weeks post-trauma. Moreover, during this time frame we additionally assessed the classical ventilatory parameters of the animal during eupnea and the EMG of multiple inspiratory muscles including the diaphragm, external intercostals, and genioglossus. These data enabled us to comprehensively assess the degree of respiratory motor deficit evident following mid-cervical contusion in a pre-clinical animal model, assess how any such deficit is altered from acute to chronic stages after injury, and quantify the degree of functional endogenous, reparative, or compensatory plasticity evident within the system.

2. Materials and methods

All experiments were approved by the Institutional Care and Use Committee at Case Western Reserve University, Cleveland. Animals were housed in groups of three, exposed to a normal dark-light cycle with free access to food, water, and environmental enrichment ad libitum. The health and welfare of the animals was monitored by the study investigators and veterinary staff at Case Western Reserve University.

2.1. Surgical procedures and diaphragm electromyography (diaEMG) recordings

2.1.1. Left lateral C3 contusion

Adult male Sprague Dawley rats (334.9 ± 3.48 g; $n = 52$; Harlan Laboratories Inc., Indianapolis, IN, USA) were anesthetized through intraperitoneal (i.p.) injection with a ketamine/xylazine cocktail (70 mg $\text{kg}^{-1}/7$ mg kg^{-1}). Upon reaching a surgical plane of anaesthesia, animals were shaved and the dorsal neck cleansed with Betadine and 70% ethyl alcohol. Carprofen (5 mg/kg) was administered through subcutaneous (s.c.) injection while 0.002% bupivacaine hydrochloride injections were applied along the site of incision. Body temperature was maintained throughout surgery at 37 ± 1 °C. A dorsal midline incision, ~3 cm in length, was made over the cervical region and the skin and paravertebral muscles retracted. A laminectomy was performed over C2 and C3 exposing the spinal cord but keeping the dura intact. Animals were placed in the Infinite Horizon contusion impactor (Precision Systems and Instrumentation) with clamps placed around the C2 and C3 lamina to stabilise the cord during the injury. Animals received a 150 kD left lateral contusion with zero dwell time ($n = 27$). The

anatomical completeness of the injury was confirmed through microscopy following cresyl violet staining. All procedures were performed on sham injured animals ($n = 26$), apart from the contusion. No animals died following the surgery, although contused animals typically required 10 mins of manual ventilation following the initial trauma. A subcutaneous injection of yohimbine (1.2 mg/Kg, Tocris) was administered to reverse the effect of the respiratory depressant xylazine. The muscle layers were sutured together (3-0 vicryl) and the skin closed using wound clips. Following surgery, animals were given buprenorphine (30 $\mu\text{g}/\text{kg}$) and saline subcutaneously. Analgesics were maintained up to five-days post-surgery.

2.1.2. EMG recordings and right lateral C2 hemisection

At pre-determined end points, animals were anesthetised through i.p. injection of urethane (1.6 mg/kg). Upon reaching a surgical plane of anaesthesia, animals were shaved and the dorsal neck cleansed with Betadine and 70% ethyl alcohol. 0.002% bupivacaine hydrochloride injections were applied along the site of incision. Body temperature was maintained throughout surgery at 37 ± 1 °C. With the animal in prone position, a 5-cm laparotomy was performed to expose the abdominal surface of the diaphragm. Further, lateral 2 cm incisions were made over the left and right rib cage and the latissimus dorsi bluntly dissected to expose the external intercostals at T1 and T2. Finally, a 1 cm midline incision was made down the animal's throat. The digastric muscle was retracted to expose the genioglossus. Bipolar platinum electrodes (Grass Technology, Middleton, WI, USA) were placed in the crural region of the left and right hemidiaphragms (dorsal to the anterolateral branch of the inferior phrenic artery), into the left and right external intercostals at T1, and into the left genioglossus muscle. Activity was amplified (gain 5000 \times ; Quad-P511 Amplifier; Grass Technology), band pass filtered (30–3000 Hz; Grass Technology), digitized, and recorded using a data acquisition system (CED1401; Spike2; Cambridge Electronic Design). The integrated signal was rectified and smoothed at a time constant of 0.075 s. All recordings were obtained during eupnea prior to a 20-s nasal occlusion.

As described previously (Awad et al., 2013), during end-point EMG recordings, a lateral right C2 hemisection was performed in order to remove descending input to the contused phrenic motor pool from the contralateral uninjured side. A dorsal midline incision ~3 cm in length was made over the cervical region and the skin and paravertebral muscles were retracted. Using micro scissors, the C2/3 spinal cord was re-exposed and durotomy performed. Using a 21G needle (with the tip positioned towards the mid-line), a right lateral hemisection was performed caudal to the C2 dorsal roots. The incision was made till the needle had reached the ventral lamina surface. This process was repeated five times and extended from the midline to the most lateral extent of the spinal cord. The functional completeness of the injury was confirmed through absence of activity in the right diaEMG.

Time to cease left diaEMG activity was calculated from the last breath prior to completing the hemisection to the final peak on the integrated response. Statistical analysis was performed using a one-way analysis of variance (ANOVA) with post-hoc Bonferroni (SPSS). Divergences were considered significant if $p < 0.05$. Data are presented as mean \pm SEM. At the time of recording and analysis, investigators were blind to the treatment group of each animal. A total of 43 animals underwent terminal EMG recordings ($n = 22$ contusion; $n = 21$ sham).

2.2. Whole body plethysmography (WBP) recordings

Individual animals were placed in Perspex plethysmography chambers (DSI). Airflow within the chamber was maintained at 2 L/min using a flow controller (DSI). Prior to each recording, the chambers air-tight seal was assessed and air volumes calibrated (DSI) which was used to calculate tidal volume (VT) changes in respiratory activity. Animals were acclimated to the chambers by placing them in the closed

environment for increasing lengths of time for five days prior to the first recording. The animals core body temperature was assessed at the conclusion of each experiment using a rectal thermometer (Physitemp). Measurements were continuously recorded through the proprietary Pomenah software (DSI). Animals were placed in the chambers for 100 mins, the first 60 of which acted as an acclimation period and was never analysed. Through the remaining 40 mins, rates of ventilation (VE) through normoxia (normal air) were assessed. Recordings were performed one day prior to the contusion (baseline), three days following injury, and then weekly up to twelve weeks post trauma. Baseline recordings were not statistically different from those conducted during the acclimation for each animal ($p = ns$) indicating the stability of the baseline. Statistical comparisons were made between treatment groups and within plethysmography measurements using one-way or two-way, repeated-measures ANOVA (SPSS). Divergences were considered significant if $p < 0.05$. Data are presented as mean \pm SEM. At the time of recording and analysis, investigators were blind as to the treatment group of each animal. 20 animals underwent WBP recordings ($n = 10$ sham, $n = 10$ contusion). This includes the 5 sham and contusion animals that additionally had EMG recordings.

2.3. Data analysis and ventilatory pattern variability

The raw plethysmography data was transferred to a custom software program (Case Western Reserve University) and assessed in three 60 s epochs taken at ~70, 80, and 90 mins into the recording while the animal was at rest. Animals were monitored during the recording to verify their physiological state (i.e. relaxed, walking, sniffing, grooming). For each epoch, the software system identified discrete breaths which were manually verified and then data averaged for each animal.

As described previously (Chung et al., 2013; Dhingra et al., 2011), Mutual Information (MI) and Sample Entropy (SampEn) of raw and surrogate data were determined. Surrogate data for MI and SampEn were computed as previously described (Chung et al., 2013; Dhingra et al., 2011). Schematic diagrams describing the acquisition of data for both MI and SampEn are shown in (Dhingra et al., 2011). MI is an expression of the degree to which knowledge of a specific point in a data set $x(t)$ reduces the uncertainty associated with a time advanced coordinate $x(t + \tau)$, where $\tau > 0$ is the time interval between samples (determined by sampling frequency). It is assessed for each time lag (τ) from unity to one respiratory cycle length. In essentials, MI provides a measure of the statistical dependence between points in the data set and includes both linear and nonlinear correlations (Fraser, 1986; Shannon, 1997). A decrease in value represents reduced complexity and greater variance in a respiratory waveform.

SampEn is calculated through the creation of templates for each point (template = m points and $m + 1$ points separated by τ). A match is a point within a tolerance interval r . SampEn defines the negative natural logarithm of the conditional probability that a specific epoch will have the same number of matches for m and $m + 1$ number of points. We performed the assessment with $m = 2$ and $r = 0.2 * SD$. It is assessed for τ from unity to one cycle length with resulting values averaged. Values with high linear correlations were excluded (defined by the first minimum of the mutual information function). As such, SampEn measures temporal pattern variability, indicative of self-similarity within a given time frame. High values convey reduced self-similarity and predictability with greater complexity (Richman and Moorman, 2000). The nonlinear complexity index (NLCI) was calculated as the average of the statistically significant differences between the original and surrogate SampEn data sets at each τ . The higher the value of the NLCI, the greater the contribution of nonlinear and/or non-Gaussian sources of variability within the data set.

All experiments were assessed blind. A minimum of 3 repeats were conducted for each experiment. The parameters were compared between control and the test groups by repeated measures two-way

analysis of variance (ANOVA; SPSS). Significance values represented as $* = p < 0.05$, $** = p < 0.01$, $*** = p < 0.001$. Data are presented as mean \pm SEM.

3. Results

3.1. Unilateral contusion of the spinal cord

Contused animals received an average 218.3 ± 16.2 kDyne injury with $1546 \pm 42.2 \mu\text{m}$ displacement. There were no deaths during or after any of the surgical procedures and no animal showed signs of blood in their urine or stool. While contused animals did show an initial decrease in weight 3 days following injury, this was recovered by 1 week after trauma (Fig. 2A). Moreover, regardless of group, all animals showed weight increase from pre-injury baseline ($F(1,252) = 22.3$, $p < 0.0001$). Sham and contused animals show no divergence in weight gain in the weeks following injury (Fig. 2A). This would suggest that alterations in weight are not a causal factor in any of the effects described.

3.2. Contusion injury causes a robust deficit in tidal and minute volume

In order to assess the effect that contusion injury had upon ventilatory parameters, the frequency (F), minute (VE) and tidal (VT) volume were assessed in eupnea weekly from acute to chronic stages following trauma (Fig. 1; Supplementary Table S1). Baseline values between groups for F, VE and VT were not statistically different ($p = ns$; Supplementary Table S1). Data show VE ($F(1,252) = 49.82$, $p < 0.0001$) and change in VE ($F(1,252) = 128.78$, $p < 0.0001$) statistically vary between our treatment groups and that this difference in response does not change with time (respectively: $F(1,252) = 0.89$, $p = 0.562$ and $F(13,252) = 1.45$, $p = 0.952$). Specifically, we demonstrate that contusion injury causes a robust decrease in change of VE from the acute stages after trauma as compared to both pre-injury baseline and sham controls (Fig. 1A). This would suggest that our contused animals exchange a smaller volume of air with time compared to baseline and sham controls, likely increasing blood carbon dioxide levels. This deficit is robust, lasting up to twelve weeks after the initial impact.

The change in VE exhibited through our data set is primarily caused by the significant decrease in VT ($F(1,252) = 49.82$, $p < 0.0001$) and change in VT ($F(1,252) = 175.63$, $p < 0.0001$) following contusion injury (Fig. 1B; Supplementary Table S1). These alterations are constant with time up to twelve weeks following injury (respectively: $F(1,252) = 0.89$, $p = 0.562$ and $F(13,252) = 0.62$, $p = 0.834$). This would suggest that our contused animal's breath a smaller volume of air with each breath compared to baseline and sham controls. While shams typically show an increase in VE and VT as compared to baseline, this is not typically statistically significant (Fig. 1A, B). Interestingly, we do not see such deficits in frequency of breaths following contusion injury (Fig. 1C; Supplementary Table S1). While there are significant differences between our treatment groups for both frequency of breaths ($F(1,252) = 13.72$, $p = 0.0003$) and change in breath frequency ($F(1,252) = 52.4$, $p < 0.0001$), these vary with time (respectively: $F(1,252) = 3.41$, $p < 0.0001$ and $F(13,252) = 2.6$, $p = 0.0021$). Contused animals show an increase in frequency change which peaks a month following the initial trauma, but then slowly decreases to baseline levels. Sham injured animals, demonstrate a gradual decrease in respiratory frequency nine weeks following the initial surgery (Fig. 1C; Supplementary Table S1). These alterations in breathing frequency suggest that endogenous changes are happening following trauma. However, collectively these data signify that any endogenous recovery which occurs within the respiratory motor system following mid-cervical contusion is not sufficient to mitigate the great effect the injury had upon ventilatory parameters. These data exhibit a profound deficit caused by mid-cervical contusion that lasts from acute to chronic

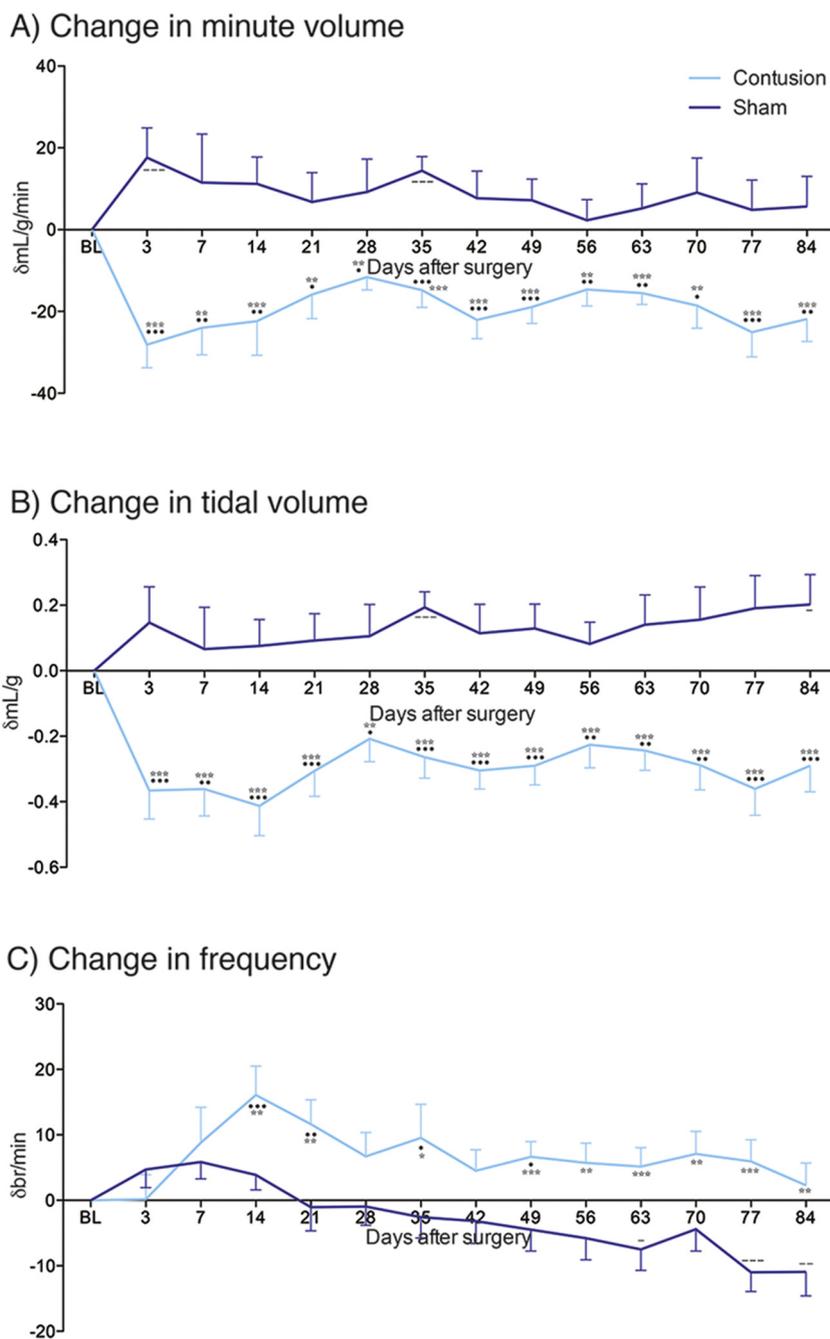


Fig. 1. Contusion injury causes profound and robust deficit in respiratory parameters. Changes in A) minute volume (VE), B) tidal volume (VT), and C) frequency over time following pre-trauma baseline (BL), C3 contusion injury (light blue), and sham control (blue). Graphs show means ± SEM. Comparisons between BL and contusion • = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$; BL and sham - = $p < 0.05$, - = $p < 0.01$, --- = $p < 0.001$; contusion and sham * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

time points.

3.3. Contusion injury causes deficits in ventilatory pattern variability

To further assess the effect of contusion injury on respiratory parameters, the ventilatory pattern variability of the waveforms from each animal were assessed (Fig. 2). From one week post-surgery, MI was shown to increase in contused animals alone as compared to baseline (Fig. 2C). Sham injured animals showed no such increase in MI at any time-point following surgery. These alterations between treatment groups were significant ($F(1,252) = 20.44, p < 0.0001$) and robust over the 12 week time frame ($F(13, 252) = 1.44, p = 0.142$), with no changes shown in the data-sets over this period. For surrogate data

sets, MI was shown to have a similar pattern as that exhibited for the original data set (Fig. 2C). Namely, MI showed a significant increase compared to baseline from three days' post trauma for the contused animals alone ($F(1,252) = 33.8, p < 0.0001$). No difference was shown in sham injured animals. These alterations in MI for the contused animals were robust over time, showing no statistical variance in the data set ($F(13,252) = 1.38, p = 0.168$). These data identify that contusion injury causes a robust increase in the statistical dependence between points in the plethysmography waveform. This suggests that the contusion injury altered the ventilatory pattern, with reduced variability that persisted up to twelve weeks.

SampEn was shown to significantly decrease in animals with contusion injury as compared to baseline (Fig. 2D; $F(1,252) = 24.44,$

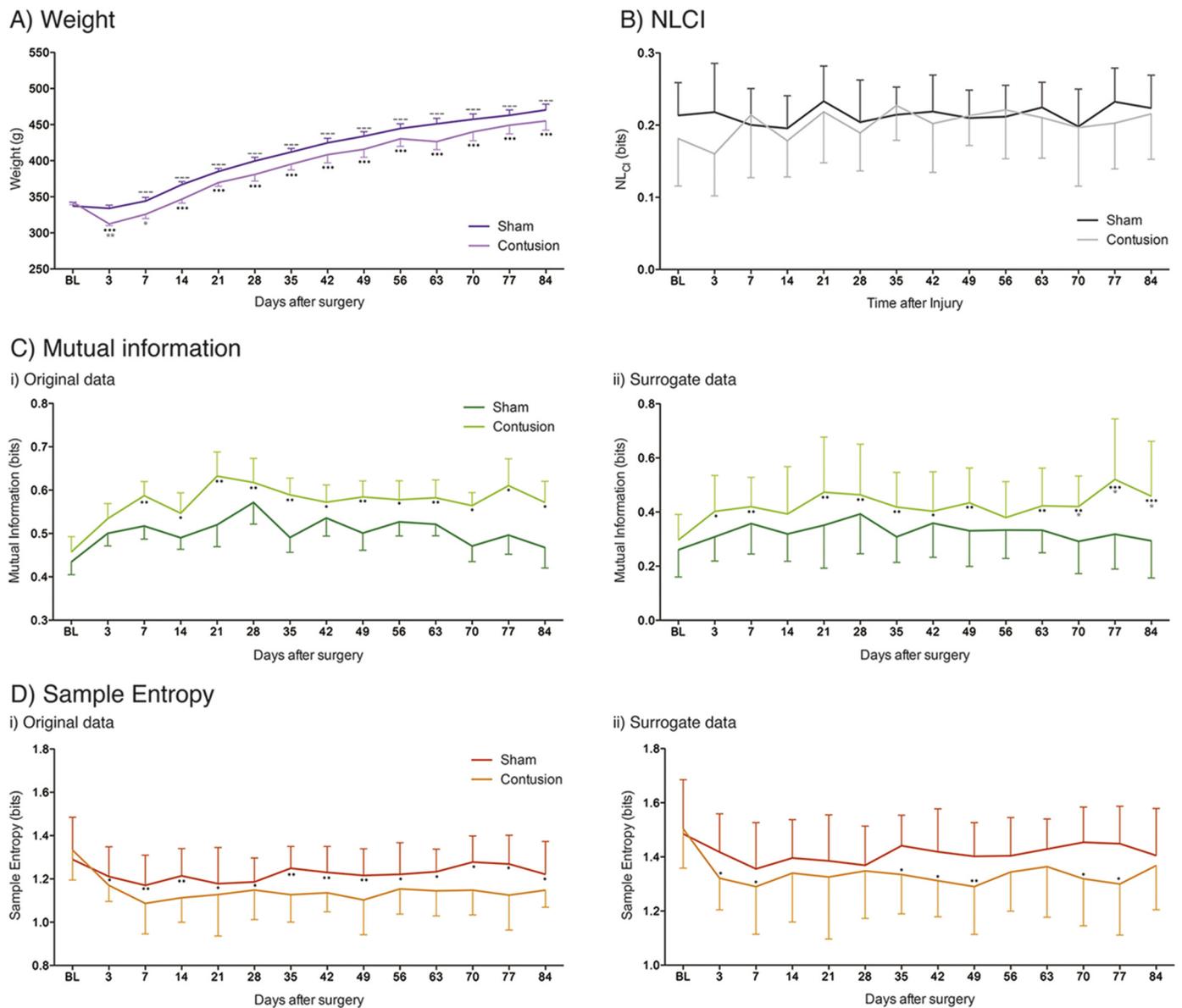


Fig. 2. Contusion injury causes profound and robust decrease in linear ventilatory pattern variability. A) Contused animals (pink) show minimal deficit in weight compared to sham controls (purple) up to twelve weeks following trauma and weight gain compared to baseline (BL). B) Contusion (light grey) and sham (tungsten) injury do not cause an increase in nonlinear variability shown through the nonlinear complexity index (NLCI) However, C) Mutual Information, and D) Sample Entropy of i) the original and ii) surrogate data sets following pre-trauma baseline (BL), C3 contusion injury (light green/orange), and sham control (dark green/red). Graphs show means \pm SEM, Comparisons between BL and contusion * = $p < 0.05$, ** = $p < 0.01$; *** = $p < 0.001$; contusion and sham * = $p < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

$p < 0.0001$). Sham injured animals showed no such decrease in SampEn at any time-point following surgery. Similar to MI, this response was shown to be robust from acute to chronic time points post injury, lasting up to twelve weeks following contusion. Interestingly, the effect of time is statistically significant on this data set ($F(13,252) = 1.96, p = 0.0241$). For surrogate data sets, SampEn was shown to have a similar pattern as that exhibited for the original data set (Fig. 2D). Differences between the treatment groups were determined to be statistically significant ($F(1,252) = 15.53, p < 0.0001$), and these alterations robust with time ($F(13,252) = 1.18, p = 0.296$). However, post-hoc tests do not show a robust decrease in the SampEn of contused animals at every time point following injury. Collectively, these data suggest that contusion injury causes a reduction in temporal pattern variability within the respiratory waveform. As such, following the contusion injury, the waveform is more self-similar and predictable with greater complexity but less variance.

To determine the source of the differences in variance shown in our data sets in response to contusion injury the NLCI was determined. Differences between the SampEn of the original and surrogate data sets were used to assess and quantify the NLCI of the breathing patterns for each treatment group (Fig. 2B). NLCI was shown to not statistically alter between treatment group ($F(1,252) = 3.13, p = 0.0782$) or change with time ($F(13,252) = 0.99, p = 0.466$). This suggests that the changes in variance shown in our data in response to contusion injury are largely due to linear/Gaussian sources. Together, these data suggest that a mid-cervical contusion injury causes a decrease in linear sources of variability within breathing patterns which is permanent, showing no endogenous recovery with time.

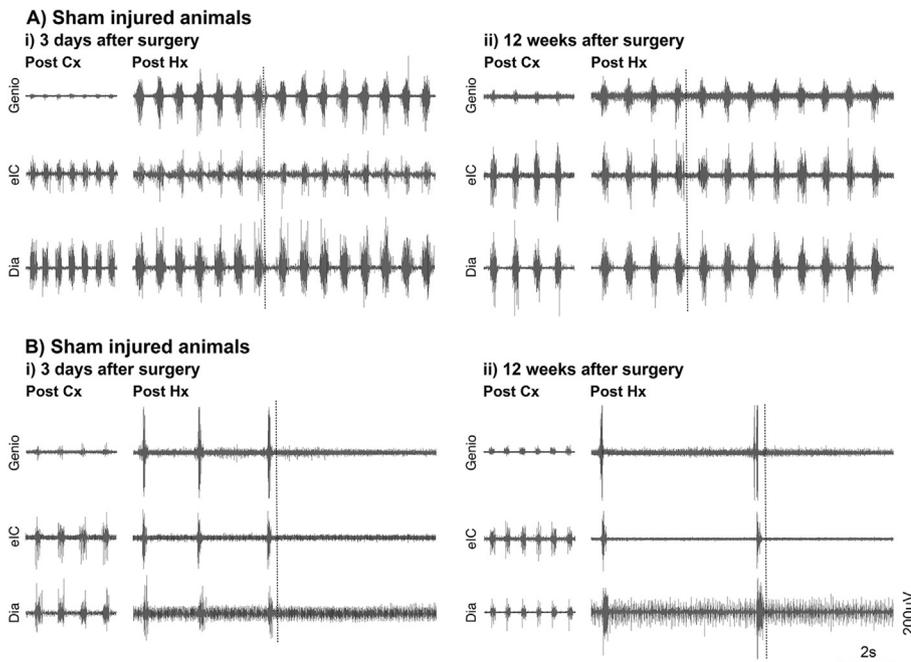


Fig. 3. Endogenous recovery is not sufficient to mediate functional activity through the contused bulbospinal pathways at both acute and chronic time points post injury. EMG data is shown for the diaphragm (dia), external intercostals (eIC) and genioglossus (genio) ipsilateral to the A) sham or B) contusion injury i) 3 days and ii) 12 weeks after C3 contusion. While recovery in ipsilateral diaphragm activity following contusion (Cx) is evident at chronic stages it is not sufficient to maintain that activity when ipsilateral pathways are isolated through right contralateral hemisection (Hx; dashed line), regardless of time post injury. This effect is evident in all inspiratory muscles. All panels are recorded in the same animal.

3.4. Endogenous recovery is not sufficient to aid respiratory recovery through contused pathways

To further assess the effect of contusion injury upon respiratory motor function we evaluated the EMGs of a number of inspiratory muscles at acute (three days) and chronic (twelve weeks) time points post injury (Fig. 3). Sham injured animals show strong ipsilateral hemidiaphragm and external intercostal activity at both three days and twelve weeks' post-surgery (Fig. 3A). The activity of the genioglossus is indicative of moderate respiratory drive. Contused animals show significantly reduced diaphragm EMG activity which appears to diminish in amplitude between three days and twelve weeks' post injury (Fig. 3B). The strong activity exhibited in the ipsilateral external intercostals during this time frame demonstrates that the multitude of decussating pathways which innovate the intercostal motor pool from the contralateral side of the spinal cord are still functioning appropriately, and they are partially compensating for the reduced activity of the ipsilateral hemidiaphragm (Fig. 3B). Similar to sham animals there is also little activity in the genioglossus when breathing during eupnea. Moreover, there is no difference in the EMG of these respiratory muscles from acute to chronic time points following injury (Fig. 3B). This would suggest that little endogenous recovery has occurred to facilitate a recovery of respiratory motor function over time.

In order to determine the extent to which any recovery occurred through the contused bulbospinal pathways at acute to chronic time points following injury a C2 hemisection was performed on the opposite side of the spinal cord to the original injury (Fig. 3). This removed any descending contralateral influence to the muscles under investigation (Awad et al., 2013; Baussart et al., 2006). Sham injured animals that underwent this procedure showed a compensatory increase in the amplitude of muscle EMG (particularly the genioglossus) at both acute and chronic time points post injury following completion of the hemisection (Fig. 3A). Indeed, animals continued to breath up to the maximal time under assessment following removal of the contralateral respiratory activity (Table 1). Spinal or neurogenic shock is likely to be the causal factor in any sham animal which stopped breathing during this time frame and all animals could be resuscitated if respiration failed during this time.

Following completion of the contralateral hemisection in animals with a prior contusion injury, a striking activity profile is seen

compared to that of the sham group (Fig. 3B). Prior to completion of the hemisection there is typically strong activity in all of the ipsilateral respiratory muscles, perhaps indicative of an increase in drive. However, upon completion of the ipsilateral hemisection the animal typically stops all respiratory activity (Fig. 3B). This is even seen in the genioglossus, a muscle innervated above the level of injury. This would suggest considerable respiratory motor feedback is mediating pattern generation during this period. The near immediate cessation in all respiratory motor activity following hemisection was seen from acute to chronic time points in contused animal. While the average length of time animals continued to breath marginally increased with time post injury (Table 1), perhaps indicative of some endogenous recovery, this was not significant. Further, all times were significantly lower than that of the sham animals at all time points ($F(11,50) = 16.63, p < 0.0001$). None of these contused animals could be resuscitated once breathing had stopped. Collectively these data suggest that marginal endogenous recovery occurs to the respiratory motor system following mid-cervical contusion injury. However, this is not sufficient to mediate extensive breathing function either when offset by influence from the contralateral side of the cord or solely through the damaged ipsilateral bulbospinal pathways alone.

4. Discussion

The present study demonstrates long-term deficits in both VE and VT during eupnea caused by unilateral mid-cervical contusion injury as assessed through WBP. Further, ventilatory patterns of contused animals show a significant increase in Mutual Information and decrease in SampEn. This reduction in variability is primarily linear in nature and suggests that these animals are likely less able to adapt to alterations in activity or changes in their surrounding environment. These effects are evident from acute to chronic stages following the initial trauma, demonstrating the great effect mid-cervical contusion has upon ventilatory activity. Finally, we establish a pronounced deficit in ipsilateral diaphragm EMG activity due to the contusion injury, becoming more pronounced at chronic stages. Conversely, the activity of the ipsilateral external intercostal muscle did regain function within three days post-injury, demonstrating substantial compensatory plasticity which lasted up to twelve weeks following trauma. However, without contralateral descending input, activity in all respiratory muscles ceased, indicating

Table 1

Time to cease EMG activity following completion of the contralateral C2 hemisection. Time given in seconds. Recordings were terminated at 5 min after completion of the hemisection. ANOVA shows a significant main effect ($p < 0.0001$; $F(11, 50) = 16.63$) with the sham data showing a significance difference to the contusion data at all time points with a minimum of $p = 0.05$.

	Days post <i>contusion</i> injury					Days post <i>sham</i> injury				
	3	7	21	42	84	3	7	21	42	84
<i>n</i>	4	5	4	4	4	4	4	4	4	5
Mean (sec)	0.0	6.757	8.981	12.13	21.14	262.2	281.3	300.0	300.0	196.8
SEM	0.0	6.757	8.981	12.13	12.43	37.77	18.7	0.0	0.0	63.93
Min	0.0	0.0	0.0	0.0	0.0	148.9	225.2	300.0	300.0	11.38
Max	0.0	33.79	35.92	48.52	48.07	300.0	300.0	300.0	300.0	300.0

little endogenous recovery occurs through the contused respiratory pathways regardless of time post-injury and the negative effect of mid-cervical contusion on afferent feedback. These data show that mid-cervical contusion can result in extensive deficits throughout the respiratory motor system. That, while plasticity is evident, there is little evidence of endogenous recovery. Functional shortfalls impact clinical outcome measures from acute to chronic time points.

4.1. Mid-cervical contusion injury

There is a division within the field regarding the nature of respiratory function following mid-cervical contusion injury. While, a number of studies show some degree of deficit within motor function, such as phrenic nerve output or diaphragm EMG amplitude following trauma, this is often transient (Baussart et al., 2006; el-Bohy et al., 1998; Stamegna et al., 2011; Wen and Lee, 2017). Moreover, compromise to ventilatory parameters is often limited to the first few days following injury (Alvarez-Argote et al., 2016; Awad et al., 2013; Golder et al., 2011; Lane et al., 2012; Nicaise et al., 2013). There are notable exceptions to these generalisations. However, we have uniquely shown that mid-cervical contusion causes a decrease from baseline in both VT and VE, a reduction in ventilatory pattern variability, and diminished ipsilateral diaphragm function, none of which recover with time. These data suggest our cervical contusion model causes a deficit in ventilation and respiratory motor control which persists from acute to chronic stages of injury.

We suggest that three factors influence the longevity of the respiratory deficit produced. First, the configuration of our animal at the point of injury ensures considerable stability of the spinal cord and column (Lee et al., 2017) and, with the pia removed (Baussart et al., 2006), guarantees the production of a severe cervical SCI with little variance between animals (Awad et al., 2013). Despite the severity of the injury and effects produced, the unilateral nature of the contusion ensured the animals survival without respiratory assistance or obvious bladder or bowel problems meaning we can produce clinically relevant effects on the respiratory motor system without adversely affecting the animals quality of life. Second, recovery could be subject to sex and strain differences. For example, following cervical hemisection, female rats demonstrate superior recovery of VT and phrenic nerve activity under challenge than males, conceivably due to alterations in hormone levels aiding respiratory responses (Doperalski et al., 2008). The use of male Sprague Dawley rats in this study could prolong the respiratory deficit produced. Third, it is possible that models of mid-cervical contusion performed by other investigators are activating spared or latent tissue, regaining ipsilateral functionality. Due to the bilateral innervation of the phrenic motor neurons from the premotor neurons of the rostral ventral respiratory group (Lipski et al., 1994) it is possible that either the contralateral descending pathway or crossed spinal pathway are activating residual ipsilateral phrenic motoneurons, supporting ventilation and respiratory motor function. We see little evidence of this in the present study except, perhaps, in conditions of extreme respiratory drive through ipsilateral diaphragm EMG recordings.

Nonetheless, the significant and robust deficit in ventilation and respiratory motor function we obtain following our model of mid-cervical contusion is an advantage in both accurately mimicking the respiratory effects of clinical cervical contusion and providing a baseline from which to address recovery following trauma.

4.2. Reductions in ventilatory parameters and variance of contused animals

We demonstrate a transient increase in respiratory frequency in response to mid-cervical contusion injury. This short-term alteration in response to the injury has been demonstrated in similar contusion models (Golder et al., 2011; Lee and Kuo, 2017; Nicaise et al., 2013), although we illustrate that this effect occurs for longer than shown previously (seven rather than two weeks). The effect is likely caused by alterations in pathways affecting afferent feedback and chemoreflexes (Golder et al., 2011; Lee and Kuo, 2017) or due to an increase in compensatory drive from the supraspinal pathways (Choi, 2005). Interestingly, we do see profound decreases in VT and VE in eupnea which are constant from three days to twelve weeks following mid-cervical contusion. Ventilatory parameters have either been shown not to change following contusion (Alvarez-Argote et al., 2016; Lane et al., 2012) or to recover in the first few weeks following mid-cervical trauma (Choi, 2005; Nicaise et al., 2013). In support of our study, Stamegna et al. (2011) demonstrated a reduction in eupnea VE in animals following mid-cervical contusion 3.5 months following the initial trauma. Moreover, Lee et al. (2017) established that both VE and VT were decreased one month subsequent to the injury. Further, significant reductions in VT following cervical contusion have been shown to remain constant for eight weeks following the surgery, although VE was only similarly affected at acute stages (Lee and Kuo, 2017; Wen and Lee, 2017). Similar to transection models (Fuller et al., 2006, 2009; Golder et al., 2001; Goshgarian et al., 1986), the reduction in ventilatory parameters exhibited within this study is likely caused by significant damage to unilateral cervical white matter at, and rostral to, the injury site (Lane et al., 2012) damaging the bulbospinal pathways responsible for inspiratory activity. Moreover, this would further the effect of phrenic motor neuron (Alvarez-Argote et al., 2016; Golder et al., 2011; Lee et al., 2017; Lee and Kuo, 2017) and diaphragm neuromuscular junction (Alvarez-Argote et al., 2016; Nicaise et al., 2013) loss typified by this injury. This lack of innervation causes muscle atrophy and decreases in muscle fibre conduction velocity, diameter, and isometric force (Gill et al., 2014; Smuder et al., 2016; Wu et al., 2014) which are not recovered upon potential reinnervation (Nicaise et al., 2013; van der Meulen et al., 2003). While there is potential for compensatory plasticity originating from the contralateral respiratory motor pathways to augment ventilatory parameters (Lee et al., 2017), this does not occur within our model.

Further to the robust decrease in VT and VE demonstrated, we have uniquely shown that the variance of ventilatory patterns is also compromised from acute to chronic stages following mid-cervical contusion. This is illustrated through an increase in the statistical dependence between points on the waveform and a decrease in self-similarity of the

ventilatory pattern waveform (Dhingra et al., 2011). These data suggest that animals display a more regular breathing pattern following mid-cervical contusion injury. Evaluation of surrogate data sets reveals this decrease in ventilatory variance to be caused by the contribution of linear Gaussian stochastic sources of variability. These differences are unlikely to be caused by alterations in respiratory rate, as this changed over time for each treatment group following the injury. One could hypothesise that reductions in ventilatory variance would be caused by similar means to that which affects ventilator parameters (namely denervation of nerves or muscle which cannot be compensated for through induction of plasticity) although this physiological explanation has not been proved within the current study. Nonetheless, the robust decrease in ventilatory pattern variability shown may further explain why previous investigators have demonstrated the inability of contused animals to respond to hypoxic or hypercapnic conditions (Alvarez-Argote et al., 2016; Golder et al., 2011; Lane et al., 2012; Stamegna et al., 2011). Similarly, the hypercapnic response is blunted in humans with cervical SCI (Lin et al., 1998; Manning et al., 1992). The reduction in pattern variability is likely a manifestation of an injury respiratory control system that is unable to adequately respond to changes in environment or activity. As such it is a highly significant and clinically relevant measure of respiratory function following cervical SCI.

4.3. Compromised diaphragm activity from acute to chronic time points

Similar to the robust decrease in ventilatory parameters, we find that the mid-cervical contusion causes a substantial impairment of the ipsilateral hemidiaphragm which lasts from three days to twelve weeks following the injury. Sham injured animals displayed strong activity in both the ipsilateral diaphragm and external intercostal EMG during eupnea with little variation. These data are regularly confirmed in similar mid-cervical contusion models (Alvarez-Argote et al., 2016; Baussart et al., 2006; Mantilla et al., 2011; Stamegna et al., 2011; Wen and Lee, 2017). Indeed, even after the advent of the contralateral C2 hemisection, only small increases in amplitude of the diaphragm are noted (Baussart et al., 2006). The compensatory plasticity within the intact system is sufficient to mediate respiratory activity.

A distinctly different effect on muscle EMG function is caused following mid-cervical contusion. We show that ipsilateral diaphragm activity remains minimal from acute to chronic time points after trauma during eupnea. This activity may be mediated through either, or both, ipsi- or contralateral innervation and has been demonstrated in a selection of similar contusion models. For example, Baussart et al. (2006) showed minimal ipsilateral diaphragm EMG activity up to one month following contusion injury. Negligible recovery, or compensatory function, was demonstrated at three months following trauma in the same model (Stamegna et al., 2011). Wen and Lee (2017) showed an initial decrease in diaphragm EMG activity which partially recovered at eight weeks following injury. Further functional impairment following mid-cervical contusion has been shown through assessment of phrenic nerve activity (Baussart et al., 2006; el-Bohy et al., 1998; Golder et al., 2011; Lane et al., 2012; Nicaise et al., 2012, 2013). For example, Nicaise et al. (2012, 2013) showed that immediately after contusion phrenic nerve compound muscle action potential was decreased and remained so for two weeks. However, some studies have shown a minimal effect of contusion on ipsilateral diaphragm EMG output when the data was normalised to maximal output (Alvarez-Argote et al., 2016; Rana et al., 2017). Although this may be limited by electrode placement within the diaphragm (Baussart et al., 2006; Li et al., 2015), their use of indwelling electrodes may suggest that the data in our study could be confounded through the use of anaesthetic at the time of recording (Chung et al., 2013; Lee and Kuo, 2017). The reduction in diaphragm EMG activity following spinal contusion is likely mediated through injury-dependent loss of ventral horn grey matter, and thus the phrenic motor neurons and propriospinal interneurons (Lane et al., 2012).

In our model, compensatory activity allows for an increase in diaphragm EMG function when the respiratory motor system is further compromised (e.g. performing a contralateral C2 hemisection) which is mediated through the contralateral, decussating pathways (Huang and Goshgarian, 2009; Lipski et al., 1994). The contused, ipsilateral pathways do not recover sufficient function at either acute or chronic time points following injury to alone cause respiratory activity. The lack of restorative plasticity within our model is likely caused by the position of our uni-lateral mid-cervical contusion with the impact affecting both the neurons in the phrenic motor pool and the bulbospinal pathways (Baussart et al., 2006). Conversely, we demonstrate substantial compensatory activity in the external intercostals ipsilateral to the contusion, which regain and retain function from three days to twelve weeks following trauma. These data confirm the findings of previous studies (Wen and Lee, 2017). The recovery in activity of the ipsilateral external intercostals is more rapid than the seven-fourteen day time-lag shown following C2 hemisection (Beth Zimmer et al., 2015; Dougherty et al., 2012; Navarrete-Opazo et al., 2015) and is likely mediated through propriospinal interneurons which upregulate following trauma (Lane et al., 2008).

As expected, the effect of mid-cervical contusion upon the EMG activity of the genioglossus is fairly limited as the muscle is innervated above the level of trauma. Interestingly, the muscle activity was attenuated at the point of contralateral C2 hemisection completion in previously contused animals. This occurred at all time points following trauma and would suggest that sensory feedback may be robustly altered following contusion injury due to chronic changes in respiratory circuitry (Felix et al., 2014; Lee and Kuo, 2017). However, further evidence would be required to substantiate this hypothesis. Nonetheless, this is something that needs to be assessed in greater detail due to potential clinical implications. There is substantial information to illustrate that spinal injuries cause dysfunction to the major inspiratory and expiratory muscles through both paralysis and atrophy. However, there is a deficit of information relating to how cervical SCI affects the muscles function and morphology of the upper airway. Our data suggest that this effect is substantial through modifications to sensory feedback, and may be a causal factor in the development of spinal injury associated dysfunctions such as dysphagia. Dysphagia, or swallowing difficulty, affects between 17 and 41% of SCI individuals (Lee et al., 2016) and can lead to pneumonia, airway obstruction, and chemical pneumonitis (Palmer et al., 2000). These data require substantial further assessment. Further, with a reduction in ventilatory pattern variance, dysfunction to respiratory sensory feedback could be a causal factor in the development of secondary pulmonary complications associated with SCI, such as sleep disordered breathing (SDB) and obstructive sleep apnea (Biering-Sorensen et al., 2009). This is a condition which affects ~25% of SCI patients (Fuller et al., 2013). The reduction in ventilatory pattern variability after contusion injury means that the respiratory motor system could be less likely to cope with the normal reductions in excitability and output associated with sleep. Indeed, combined with other factors demonstrated here such as the reduction in lung volume, loss of muscle function, and alterations to sensory feedback there is an increased likelihood of developing SDB following mid-cervical contusion injury. The prevalence and causal mechanisms of these secondary effects following SCI contusion models requires greater investigation to ensure that they can be better predicted and treated.

4.4. Physiological and clinical significance

Ethically and practically, it is not feasible to produce a pre-clinical model of a ventilator dependent animal (Powers et al., 2009; Sieck and Mantilla, 2008). Our model of mid-cervical spinal cord contusion (Awad et al., 2013) yields an injury with measurable and prolonged deficits in clinically relevant ventilatory parameters, variability, and motor function. As such, this model can enable the transparent and accurate analysis and evaluation of potential treatment methods for

respiratory motor function following contusion injury. Moreover, clinical outcomes to cervical contusion are variable. Patients may show evidence of endogenous plasticity and respiratory compensation, eventually being weaned from the ventilator and achieving eupnic breathing (Bluehardt et al., 1992). However, this does not occur through the restoration of pre-injury motor control, but through the introduction of accessory muscle activation (Ledsome and Sharp, 1981; Winslow and Rozovsky, 2003), which is accurately reflected within our model. In combination, these data suggest that mid-cervical contusion can produce severe deficits throughout the respiratory motor system affecting multiple ventilatory and functional outcome measures which will not recover with time unless some form of treatment is imposed.

Important to our study is the use of ventilatory pattern variability to evaluate the effect of mid-cervical contusion injury upon the respiratory system. As injury size does not predict functional respiratory outcomes in human SCI (Bluehardt et al., 1992) it is important to find other clinically relevant measures that may achieve this effect. Ventilatory variance has been shown to be an advantageous predictor of success following ventilator weaning (Seely et al., 2014; Wysocki et al., 2006). Data collection for this technique can be achieved simply and in eupnea, achieving clear indications of ventilatory deficit. We believe that this measure could be used as an ‘additional vital sign’ in the continued clinical assessment of patients with spinal cord injury, facilitating risk stratification or facilitating the development of individualised treatment strategies.

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