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Supraspinal respiratory plasticity following acute cervical spinal cord injury

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Abstract

Impaired breathing is a devastating result of high cervical spinal cord injuries (SCI) due to partial or full denervation of phrenic motoneurons, which innervate the diaphragm – a primary muscle of respiration. Consequently, people with cervical level injuries often become dependent on assisted ventilation and are susceptible to secondary complications. However, there is mounting evidence for limited spontaneous recovery of respiratory function following injury, demonstrating the neuroplastic potential of respiratory networks. Although many studies have shown such plasticity at the level of the spinal cord, much less is known about the changes occurring at supraspinal levels post-SCI. The goal of this study was to determine functional reorganization of respiratory neurons in the medulla acutely (>4 hours) following high cervical SCI. Experiments were conducted in decerebrate, unanesthetized, vagus intact and artificially ventilated rats. In this preparation, spontaneous recovery of ipsilateral phrenic nerve activity was observed within 4 to 6 hours following an incomplete, C2 hemisection (C2Hx). Electrophysiological mapping of the ventrolateral medulla showed a reorganization of inspiratory and expiratory sites ipsilateral to injury. These changes included i) decreased respiratory activity within the caudal ventral respiratory group (cVRG; location of bulbospinal expiratory neurons); ii) increased proportion of expiratory phase activity within the rostral ventral respiratory group (rVRG; location of inspiratory bulbo-spinal neurons); iii) increased respiratory activity within ventral reticular nuclei, including lateral reticular (LRN) and paragigantocellular (LPGi) nuclei. We conclude that disruption of descending and ascending connections between the medulla and spinal cord leads to immediate functional reorganization within the supraspinal respiratory network, including neurons within the ventral respiratory column and adjacent reticular nuclei.

Keywords

spinal cord injury; respiration; plasticity; phrenic; brainstem

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Introduction

Injury at nearly any spinal level can affect breathing due to the wide distribution of respiratory lower motoneurons from cervical to lumbar spinal segments (see review (Lane, 2011)). However, the most devastating effects on respiratory function arise following spinal cord injury (SCI) at high- to mid-cervical spinal levels. Among the vast range of motoneurons that become denervated following such injuries are the phrenic motoneurons (distributed from C3 to C5/6 in rats) (Goshgarian and Rafols, 1984; Kuzuhara and Chou, 1980; Lane, 2011; Lane et al., 2008; Mantilla et al., 2009), which innervate the diaphragm – the primary muscle of respiration. In the intact spinal cord, phrenic motoneurons receive bilateral input from neurons in the ventral respiratory column (VRC) as depicted in the schematic diagram in Figure 1. These projections run through lateral and ventromedial funiculi (Fuller et al., 2009; Lipski et al., 1994), and synapse onto motoneurons directly or indirectly via pre-phrenic interneurons (Lane et al., 2008).

Compromised phrenic circuitry and subsequent diaphragm paralysis typically lead to respiratory arrest and the need for assisted ventilation (Baydur and Sassoon, 2010; Como et al., 2005; Wong et al., 2012). There is mounting clinical and experimental evidence, however, for some degree of spontaneous improvement in respiratory function post-injury, reflecting the neuroplastic potential within the respiratory network. Although previous research in this field has been focused on mechanisms of plasticity within the spinal cord (Goshgarian, 2009; Lane et al., 2009; Mitchell and Johnson, 2003; Warren et al., 2013), neuroplastic changes may appear at multiple levels post-SCI including the periphery, spinal cord, brainstem, and brain. One example is the somatotopic reorganization of primary somatosensory and motor cortices after SCI as reported in many experimental and clinical studies (Endo et al., 2007; Humanes-Valera et al., 2013; Kokotilo et al., 2009; Lotze et al., 1999). Not surprisingly, neuroplastic changes in the brainstem post-SCI affecting motor control have gained increasing attention (Kumru et al., 2009; Weishaupt et al., 2013; Zorner et al., 2014). While some previous work has examined potential neuroplastic mechanisms within brainstem respiratory networks (Morris et al., 2003; Morris et al., 2000; Morris et al., 2001), few studies have focused on supraspinal plasticity following SCI (Buttry and Goshgarian, 2014; Felix et al., 2014; Golder et al., 2001; Vinit et al., 2005). For instance, previous work has found that cells within the VRC and raphe nuclei ipsilateral to a partial C2 hemisection (C2Hx) show increased Akt signaling which decreases cytoplasmic phosphorylated forkhead transcription factor (P-FKHR) immunoreactivity (Felix et al., 2014). Furthermore, phrenic motor recovery appears to be at least in part dependent on FKHR phosphorylation within brainstem respiratory nuclei (Felix et al., 2014). Studies in the neonate also revealed evidence for brainstem plasticity following C2Hx with increases in glutamatergic and adenosinergic receptors within the ipsi- and contralateral medulla (Zimmer and Goshgarian, 2007). A recent study by Buttry and Goshgarian (2014) using transynaptic WGA-Alexa 488 to trace the phrenic motor circuit shows an increased number of structures with labeled neurons in the brainstem following chronic C2Hx injury in rats. These structures include: parvocellular reticular, gigantocellular reticular, and intermediate reticular nuclei. The authors concluded that plasticity occurs at the supraspinal level to re-establish function on the injured side by the activation of normally latent compensatory

pathways, and that these pathways may be mediated by reticular nuclei (Buttry and Goshgarian, 2014). Collectively, these results show that brainstem neurons not only remain connected to the spinal cord post-injury, but they represent an unexplored therapeutic target.

Effective therapeutic targeting of brainstem neurons, however, may require a better understanding of how functional states are altered post-injury. The discharge pattern of individual brainstem neurons in pre-injury and post-injury states are still unknown. The present work is the first to examine how acute C2Hx impacts patterns of inspiratory or expiratory activity within bulbospinal neurons of the VRC and adjacent reticular nuclei. We hypothesized that interruption of bulbospinal and spinobulbar pathways directly affects the activity of ipsilateral bulbospinal and surrounding respiratory neurons within hours. The present data support this hypothesis and provide the first experimental evidence for i) reorganization of inspiratory and expiratory activity within the VRC, and ii) increased respiratory activity in the ventral reticular nuclei acutely (within hours) following cervical SCI.

Materials and Methods

Experiments were conducted on male Sprague-Dawley rats (400–450g; Harlan Laboratories, Inc.). Animals were divided into two groups: uninjured controls (n=9) and incomplete (see ‘Results’ and Figure 1C for details) C2Hx (n=8). All surgical and animal care procedures were approved by the Drexel University Institutional Animal Care and Use Committee, and adhered to AAALAC/NIH guidelines. Decerebration was used for all animals to avoid using anesthetics that significantly suppress respiratory neuronal activity, especially post-SCI. Males were chosen because they are larger than females of the same age. Larger animals have better survival rate post-decerebration and allow for easier mapping of brainstem activity.

Surgical procedures

Animals were anesthetized with isoflurane (isoflurane (4%) vaporized in O₂ (100%)) via a nose mask (Isoflurane vaporizer VIP3000, MDS Matrx, Scavenger Fluovac 50206, Stoelting). After shaving the surfaces of head, neck and ventral part of the right hind paw, each animal was placed in the supine position and an incision (2–2.5 cm) was made on the ventral side of the neck. The sternohyoid muscles were retracted and a tracheotomy was performed by inserting a tracheal cannula (polyethylene tube, 2 cm in length, OD=2.5 mm, ID=1.5mm) into the trachea and securing it with suture. After the tracheostomy, the animal was transferred to artificial ventilation (AVL, ~60 cycles per min, 3–3.3 ml tidal volume; 2% isoflurane in 100% O₂, Columbus Apparatus). Two catheters (PE-50) filled with lactated ringer’s solution and heparin (300 USP/ml) were inserted into the femoral vein and the artery for drug injections and monitoring of blood pressure, respectively. Rectal temperature was maintained at 37.0±0.5°C via a servo-controlled heating blanket (TCAT-2DF, Physitemp). To expose the left and right phrenic nerves, the ventrolateral neck muscles were gently retracted and the nerves were dissected from surrounding tissue. Nerves were cut at the distal end (just beneath the subclavian vein) and the free end of each nerve was tied with fine thread (#6-0, 1 cm length). This thread was inserted into the muscles in the dorsolateral

direction and used as a guide for retrieving phrenic nerves from the dorsal side. The internal carotid arteries were then ligated bilaterally to minimize bleeding during decerebration. Ventral neck muscles and skin were closed and sutured. Animals then were placed in prone position and the dorsal muscles surrounding the C₁–C₂ cervical segments were partially removed and retracted to enable proper placement of recording electrodes. Using the threads as a guide, phrenic nerves were gently retrieved from the ventral side. Cotton balls soaked in lactated ringer's solution were placed over the nerves and muscles to protect them from drying.

For decerebration, animals were placed into a stereotaxic frame, and dexamethasone (1mg/kg, i.m.) was injected to prevent brain edema. Arterial blood pressure and lung inflation pressure were monitored via pressure transducers (CDXII; Argon Medical) and conventional amplifiers (Gould, Ohio). End-tidal CO₂ (5–5.5%) was monitored and adjusted by frequency of ventilation (Capnostar, CWE). After biparietal craniotomies, the superior sagittal and straight sinuses were ligated, and the brain axes was transected at the rostral border of the superior colliculus. Cerebral tissue rostral to the transection was aspirated, and small pieces of gelfoam (USP, Pharmacia) soaked with a cold thrombin solution (100 USP/ml, Biopharm) were placed into the skull cavity to stop bleeding. Animals recovered for two hours after decerebration. During this time, dextrose (5% w/v in 1 ml Locke-Ringer solution) was injected intravenously (every 15–30 min) to stabilize blood pressure. Subsequently, the dorsal surface of the medulla and first two spinal segments of the spinal cord were surgically exposed. The animal was centered within the stereotactic frame, and the vertebral column was raised from the T2 vertebral spinous process to align the spinal cord and medulla. A bilateral pneumothorax was performed to eliminate artifacts associated with respiratory movement. Anesthesia was withdrawn, and a paralytic agent (vecuronium bromide, 3–4 mg/kg/h, 0.5 mg diluted in 1 ml of Locke-Ringer solution) was continuously infused (syringe pump, NE-300, New Era Pump Scientific Inc., NY) via the vein catheter. For the duration of the recording, stable end-tidal CO₂ (~5%), mean arterial blood pressure (>80 mmHg) and rectal temperature (37°C) were maintained.

Once the animal was physiologically stable, a mineral oil bath was created on both sides of the spinal cord to immerse nerves and electrodes for recording. Phrenic neurograms were recorded before, during, and after C2Hx. To limit consequences of spinal shock, animals were temporarily (10 min) placed back under isoflurane (2.5%) anesthesia during the C2Hx. The lateral hemisections were made with a scalpel blade immediately below the C2 spinal rootlets. The animal was then allowed to recover for ~4–6 hours, by which time ipsilateral phrenic activity had partially recovered spontaneously and reached a plateau.

Electrophysiological recording and Brainstem Mapping

Extracellular recordings were made with glass microelectrodes (tip diameter = 2–3 µm; 5–8 mΩ) filled with 0.5 M sodium chloride. Single and multi-unit respiratory activity was recorded at the following coordinates: from +1 (rostral) to –1.8 mm (caudal) of calamus scriptorius (CS); 1–2.4 mm from midline; and 1–3.6 mm by depth with 0.2 mm step. Recorded sites were identified as I (inspiratory), E (expiratory), or non-respiratory. Respiratory phases were determined from phrenic nerve activity recorded bilaterally with

bipolar silver electrodes (0.5 mm thickness, A-M Systems). Neuronal and phrenic nerve activity was amplified (x200–500 for neuronal and x10000 for phrenic nerve activity) and filtered (500–5,000Hz for neurons and 100–5,000 Hz for nerves) using a differential amplifier (NeuroLog, Digitimer Ltd). All signals were digitized (10 kHz sampling rate per channel) and recorded on a PC using Chart5 software (AD Instruments). Respiratory activity in the medulla (ipsilateral to the side of injury) was mapped in naive and C2 hemisectioned animals 4–6 hours following injury.

In addition to the well defined areas of inspiratory and expiratory bulbospinal neurons – the rostral and caudal ventral respiratory groups (rVRG and cVRG), respectively – our mapping sites were extended to include the nucleus ambiguus (NA), lateral reticular nucleus (LRN), ventral (MdV) and dorsal (MdD) parts of the medullary reticular nucleus, gigantocellular (Gi) and parvocellular reticular nucleus (PCRt), lateral paragigantocellular nucleus (LPGi), and intermediate reticular nucleus (IRt) (Paxinos and Watson, 2007) (see Figure 3A, B).

Histology and Immunohistochemistry

At the end of each experiment, animals were intracardially perfused with physiological saline and 4% paraformaldehyde (in 0.1M PBS). The spinal cords were dissected, post-fixed (4% paraformaldehyde), and cryoprotected (15% and 30% sucrose in 0.1M PBS, sequentially). Spinal cord tissue was sectioned on a cryostat (60 μ m thick), slide-mounted, and stained with Toluidine blue (Sigma). The extent of C2Hx was confirmed by evaluation of serial sections using bright field microscopy.

Data Analysis

Phrenic neurograms were integrated (rectified and smoothed with a 30 ms window) and onset events of phrenic activity were detected by the threshold placed above the base line using Spike2 (Cambridge Electronic Design, Cambridge, England). Integrated phrenic activity was averaged over 30 s time periods (10 min before and 10 min, 2, 4 and 6 hours post-C2Hx) and the peak magnitude was analyzed using Origin Pro 9 software (OriginLab Corporation, MA). Peristimulus time histograms (PSTHs) of single and multi-unit activity were constructed using custom made scripts in Origin Pro 9. The final classification of recorded units was made based on phrenic nerve activity that indicates inspiratory phase of respiration. 2- and 3-D colored maps of respiratory activity distribution in the medulla were built using a custom-made Matlab script (Matlab 8, The MathWorks, Inc). Types of respiratory activity (inspiratory, expiratory, and none) were defined for every 200 μ m depth at each recording site. The full map consists of 120 recording sites (points), with each point representing data from at least 2–3 animals. Percentage of respiratory activity for each point of the map (from 1 to 3.6mm depth) was calculated and color-coded: Green color represents inspiratory activity, red represents expiratory activity, and blue represents no detectable respiratory activity. Intermediate colors (between green and red) in the 2D maps reflect the relative proportion of both inspiratory and expiratory activity within the same electrode track. 3-D maps represent an expansion of these 2-D maps, each layer in the z-axis representing recordings obtained every 200 μ m (between 1 and 3.6 mm deep). The data are presented as the mean $\pm$ standard deviation (STD). For statistical analysis, a Shapiro-Wilk

test was used to test for normality and a paired t-test was applied for normally distributed data. Wilcoxon signed ranks test was used for non-normally distributed data.

Results

Acute phrenic recovery after C2Hx

Ipsilateral phrenic output was silenced acutely following incomplete C2Hx in 75% of injured animals. However, all injured animals exhibited some spontaneous recovery acutely post-injury in this unanesthetized, decerebrate preparation. Histological analysis of the lesion epicenter revealed fairly consistent sparing of dorsomedial, intermediate gray and ventromedial white matter (Figure 1C), as described previously for incomplete C2Hx (Fuller et al., 2009; Keomani et al., 2014; Vinit et al., 2007).

Representative phrenic neurograms recorded before and after C2Hx (10min, 1, 2 and 6hrs) are shown in Figure 1A. Immediately following C2Hx, no phrenic phasic activity was observed on the injured side (see Figure 1A). Within 1–2 hours post-injury, irregular non-respiratory bursts appeared. The amplitude and duration of these bursts tended to increase, merging into high amplitude tonic activity. Approximately 2–3 h post C2Hx, ipsilateral inspiratory phrenic bursts were distinguishable from tonic activity. These phasic bursts appeared with low-frequency spike rate and had a delayed onset relative to contralateral phrenic bursting. The amplitude of stable ipsilateral phrenic bursts reached a plateau about 4–6 hours post-C2Hx (see Figure 1A).

The recovery of phrenic activity over time following injury is quantified in Figure 1B, where integrated phrenic burst amplitude is shown as a percent of pre-injury amplitude. Ten minutes post-C2Hx ipsilateral phrenic bursts were detectable in only 2 of the 8 injured animals, with averaged activity $14.15 \pm 1.2\%$. Interestingly, average contralateral phrenic activity dropped to $79.5 \pm 11.2\%$ during first 10 min post-injury. Two hours after injury, average ipsilateral phrenic activity had increased ($20.7 \pm 12.7\%$) in 5 out of 8 animals, and contralateral phrenic activity reached control level ($100.8 \pm 12.2\%$). Four hours post-injury all animals recovered with average ipsilateral phrenic activity 28.6 ± 21.9 whereas contralateral phrenic activity slightly increased above the control level ($130.9 \pm 46.9\%$). The recovery of phrenic activity at 6 hours was not significantly different from 4 hours post injury ($35.7 \pm 13.8\%$ ($P=0.2$) for ipsilateral and $132.9 \pm 60.26\%$ ($P=0.9$) contralateral sides).

Mapping of respiratory activity in naïve and C2Hx animals

Maps of respiratory activity in the medulla were made using extracellular single and multi-units recordings that were classified as inspiratory, expiratory and non-respiratory related. Patterned inspiratory activity from the phrenic nerve activity was used to identify the inspiratory phase of respiration. Examples of the detectable respiratory activity recorded in our experiments are shown in the Figure 2. Intramedullary recording from uninjured animals revealed that inspiratory activity was seen primarily at the level of rVRG, located rostral to the calamus scriptorius (CS), while expiratory activity was detected mostly within the region of the cVRG, caudal to CS (Figure 3). This is consistent with previous reports of the anatomical location and relevant functions of rostral and caudal VRG (Alheid and

McCrimmon, 2008; Ezure et al., 1988; Tian and Duffin, 1996). However, some limited expiratory- and inspiratory-phase activity was detected in rVRG and cVRG, respectively. This activity recorded from a total of 120 sites in the medulla (ipsilateral to injury) is represented in a 2D-color map (Figure 3C, Control). In naïve animals, 48% of all recorded sites above CS showed patterned activity in phase with inspiration, 22% with expiration, while the remaining 30% showed no detectable respiratory-related activity. In contrast, below CS only 38% recorded sites showed inspiratory, 32% expiratory and 30% were presented with non-respiratory activity (Figure 3D).

Acutely (>4 hours) following C2Hx injury we observed a reorganization of active inspiratory and expiratory activity. Overall, there was a significant (20%; $P=0.002$) decrease in respiratory activity at the level of cVRG (below CS), with inspiratory activity at 31%, expiratory activity at 18% and non-respiratory activity at 51%. However, the number of sites recorded within the rVRG with detectable respiratory activity significantly increased (17%, $P=0.01$). This was primarily due to an increase in the proportion of expiratory-phase (36% post C2Hx vs 22% in naïve animals) activity in the rVRG (Figure 3C, D, Post-C2Hx).

Three-dimensional reconstruction (Figure 4) of these recordings offers a more detailed assessment of changes in respiratory activity both within the VRC and in surrounding structures. In both control and injured animals, the site of greatest respiratory activity was recorded at 2.7–3.0 mm depth, which corresponds to the region of the VRC - specifically the rVRG and cVRG. Following C2Hx, however, there was a significant increase in respiratory activity within more ventrally located structures (>3 mm from the dorsal surface of the medulla), which anatomically correspond to ventral reticular nuclei (LRN and LPGi). To our knowledge, this is the first demonstration of functional recruitment of reticular neurons into respiratory related activity post-C2Hx.

Discussion

Spinal plasticity within the phrenic motor system following C2Hx has been investigated since the studies of Porter (1895). While there is a growing interest in supraspinal changes following spinal cord injury (SCI), little effort has been made to explore these within the respiratory system. The present work provides the first demonstration of respiratory related functional reorganization following high cervical spinal cord injury. Using an incomplete model of lateral cervical hemisection, the results from these experiments reveal an acute shift in the pattern of inspiratory- and expiratory-related activity within the medulla following C2Hx.

Electrophysiological experiments were conducted in decerebrate, unanesthetized, vagus intact and artificially ventilated rats. This approach was selected over anesthesia, as anesthesia depresses respiratory activity and can alter tidal volume and frequency (Evers et al., 2006; Faber et al., 1982), thereby directly impacting respiratory activity within motor- and interneurons (see review (Richards, 2002)). Depth of anesthesia can change the shape and magnitude of phrenic bursts (Stuth et al., 1992), and suppress vagal and glossopharyngeal output (Ezure, 1990). In contrast, decerebration eliminates the need for anesthesia and thus the associated confounding factors. Although not extensively

investigated, however, an important consideration is that removing the forebrain (including the cortex, hypothalamus and limbic system) may also affect respiration. Regardless, decerebrate animals breathe spontaneously and respond to hypercapnia and hypoxia in a manner similar to conscious animals, which makes this model more comparable to respiration in the awake animal (Tenney and Ou, 1977).

The injury model employed in this study, incomplete C2Hx, transects bulbospinal projections from the ipsilateral VRC which descend in the lateral funiculus, but likely spares some from the contralateral VRC that descend in the ipsilateral ventromedial white matter (Darlot et al., 2012; Fuller et al., 2009; Lipski et al., 1994). Preserving these projections post-C2Hx likely plays a significant role in the ipsilateral phrenic recovery seen here (Fuller et al., 2009; Vinit and Kastner, 2009; Vinit et al., 2007). Moreover, the dorsomedial funiculus, which contains corticospinal and ascending projections to the brainstem, was also partially spared.

The loss of ascending (spinobulbar) projections to the brainstem following C2Hx has not been well characterized. C2Hx will compromise afferent feedback from sensory receptors and spino-reticular pathways which modulate brainstem activity (Sengul and Watson, 2015; Watson et al., 2009). Overall, little is known about how these ascending pathways impact the activity of the respiratory neurons in the medulla in the uninjured spinal cord, let alone following injury. However, it could be speculated that damaging these ascending pathways results in impaired feedback to brainstem neurons and a dysregulation of activity. During terminal phrenic neurograms, phrenic nerves are transected for recording purposes, which abolishes primary afferent feedback from the diaphragm in both naïve and injured animals. Future work should explore the effect that interruption of ascending spinobulbar pathways has on neuroplastic changes seen at the supraspinal level.

Acute phrenic recovery after C2Hx

Recovery of ipsilateral phrenic activity following incomplete vs. complete C2Hx has been shown in several studies (Fuller et al., 2009; Vinit et al., 2006), and is attributed to sparing of the ventromedial funiculus. In our experiments, ipsilateral phrenic activity partially recovers within hours after C2Hx, which is sooner than previously reported (Fuller et al., 2009; Vinit et al., 2008). This rapid recovery may be attributed in part to the absence of anesthesia that depresses neuronal activity, the decerebration that reduces the control of higher centers, or even the injection of dexamethasone (1mg/kg) before decerebration. For example, it has been previously shown that dexamethasone prevents brain edema, improves blood flow, and is even protective from glutamate neurotoxicity (Ogata et al., 1993).

Interestingly, the characteristics of the acute ipsilateral phrenic recovery seen in our experimental preparation develop similarly to that in chronically injured animals (Lee et al., 2013). Our data show that recovery of phrenic activity begins in late inspiration, with low-frequency discharges during a phrenic burst. Moreover, increased tonic activity in the ipsilateral phrenic nerve was seen in these early stages of recovery, similar to what was reported by Lee et al (2013) in chronically injured (C2Hx) rats. We hypothesize that increased tonic activity in the ipsilateral phrenic nerve following C2Hx reflects the disruption of inhibitory control from bulbospinal and spinal circuits and results in an

imbalance between descending inhibitory (Blessing, 1990; Holmes et al., 1994; Hossaini et al., 2012) and excitatory (for e.g., 5-HT tonic drive from raphe nuclei) inputs to phrenic motoneurons.

Medullary respiratory activity before and after C2Hx

It is widely accepted that patterns of inspiratory and expiratory activity in the rVRG and cVRG is more heterogeneous in decerebrate animals (Ezure, 1990). For instance, some expiratory-phase activity was recorded in the area above CS where inspiratory bulbospinal neurons are located (rVRG), and inspiratory-phase activity was recorded below CS in the region normally associated with expiratory bulbospinal respiratory neurons (cVRG). Following acute C2Hx, the distribution of respiratory activity in the medulla ipsilateral to injury became even more heterogeneous. An important consideration is that the decrease in respiratory activity observed in the caudal medulla (cVRG) post-C2Hx may be explained in part by its proximity to the injury site, with associated edema and spinal shock. The increased proportion of expiratory-phase activity above CS (rVRG) could be associated with a compensatory mechanism. For example, this phenomenon may reflect a rapid recruitment of these respiratory neurons into a specific post-injury breathing pattern. Alternatively, it could be attributed to compromised spinobulbar axons post-injury and dysregulation of ascending functional inputs. Another key finding from these experiments was the increased phasic respiratory-related activity seen within reticular nuclei after C2Hx. This suggests that the brainstem reticular system is involved in respiratory plasticity post-injury, seen most strikingly in the ventrolateral reticular nuclei (LRN and LPGi).

While this study has focused on functional changes within the ipsilateral brainstem, reorganization of respiratory-related activity may also arise within the contralateral brainstem. Such reorganization could result from several contributing factors. For instance, the two sides of the ponto-medullary respiratory network are anatomically and functionally integrated (Smith et al., 2013; Smith et al., 2009) and alterations in activity observed ipsilaterally likely have some effect on contralateral activity. In addition, paralysis/paresis ipsilateral to injury alters respiratory requirements, thus driving compensation and increasing activity in spared circuitry. We expect that inspiratory bulbospinal neurons in the contralateral rVRG may drastically increase their activity following hemidiaphragm paralysis, providing increased excitatory drive to the phrenic motoneurons to compensate minute ventilation (Sandhu et al., 2009). Increased inspiratory bulbospinal drive can also lead to activation of latent cross-phrenic pathway (Goshgarian, 2009) and promote recovery of ipsilateral phrenic motor output. Neurons from both the ipsi- and contralateral VRC likely contribute to this recruited CPP (Boulenguez et al., 2007; Lane et al., 2009; Lane et al., 2012; Moreno et al., 1992; Vinit and Kastner, 2009). Besides a compensatory increase of contralateral hemidiaphragm activity, other respiratory muscles can elevate their activity, including expiratory muscles (Katagiri et al., 1994; Sherrey and Megirian, 1990). Therefore, increased activity of contralateral cVRG neurons could be expected as well, in order to increase expiratory drive that also can play an important role in maintaining normal blood gas level post-injury. In addition to these functional changes, Buttry and Goshgarian (Buttry and Goshgarian, 2014) reported anatomical tracing that was consistent with bilateral recruitment of other brainstem nuclei following C2Hx.

Reticular nuclei in respiration

There is a growing appreciation in SCI literature for the role of reticular nuclei before and after injury, and their involvement in respiration as outlined in several previous studies (Andrezik et al., 1981; Li et al., 2001; Richard and Stremel, 1990). Several groups have used transynaptic tracing techniques to show anatomical connectivity between brainstem nuclei (including the reticular nuclei) and phrenic motor circuitry (Buttry and Goshgarian, 2014; Dobbins and Feldman, 1994; Lane et al., 2008; Vinit et al., 2014) indicating their potential contribution to respiration. The present work now demonstrates that the LPGi and LRN are associated with respiratory neuroplastic changes in the brainstem acutely following C2Hx. The LPGi has extensive connections with VRG and dorsal respiratory group (DRG) (Smith et al., 1989), midbrain periaqueductal gray, raphe nuclei (Lovick, 1986), the solitary tract, parabrachial, Kolliker-Fuse nuclei (Andrezik et al., 1981; Guyenet and Young, 1987; Jancso and Kiraly, 1980) and spinal cord (Andrezik et al., 1981; Liang et al., 2016). Expression of acid-sensing ion channels that were discovered in the neurons of the trapezoid body (area of the densest concentration of central chemoreceptors) also was found in LPGi neurons in the rat (Cao et al., 2009). Therefore, it was suggested that LPGi activity can be recruited during hypoxia. Overall, the literature shows that LPGi is involved in the control of homeostasis of the organism, which includes respiration (Andrezik et al., 1981; Tian et al., 1995).

The LRN is typically considered to be the relay nucleus of the spino-reticular-cerebellar tract (Payne, 1987). It receives signals from the red nucleus, cortex, fastigial nucleus and feedback projections from the spinal cord and conveys this information to the cerebellar cortex (Hryciyshyn and Flumerfelt, 1981). Spinal projections include inputs from propriospinal neurons and muscle afferents. Electrical stimulation of phrenic afferent fibers evokes responses in LRN neurons (Macron et al., 1985), indicating that the LRN receives afferent projections from the diaphragm. Electrophysiological studies confirmed that the LRN contains both locomotor and respiratory-related neurons with inspiratory and expiratory activity (Ezure and Tanaka, 1997). Some of these neurons have bimodal, locomotor-respiratory activity, suggesting their involvement in locomotor-respiratory integration (Ezure and Tanaka, 1997; Iwamoto et al., 1982). Based on previous and present work we predict that the LPGi and LRN nuclei might be involved in modulation of respiratory activity following spinal cord injury. Given that some brainstem neurons remain connected to spinal circuitry following partial SCI and appear to be involved with functional neuroplastic changes even acutely post-injury, they represent an unexplored therapeutic target. Recent studies by Vinit et al. (Vinit et al., 2016; 2014) using transcranial magnetic stimulation in the adult rat revealed that activity within brainstem pathways can not only be activated, but may be therapeutically targeted (Vinit et al., 2016; Vinit et al., 2014).

Conclusions

The current work presents the first experimental evidence of neuroplastic changes of the respiratory network at the supraspinal level immediately post high cervical SCI. At a time when modest recovery of ipsilateral phrenic activity can be observed (4–6 hours) following partial C2Hx, the distribution of detectable expiratory and inspiratory activity was altered throughout the VRC. In addition, we demonstrate the recruitment of ventral reticular nuclei

(LPGi and LRN) into respiratory networks, suggesting their role in respiratory plasticity following cervical SCI. While further investigation is required to explore the role of ipsilateral reorganization, and potential reorganization within contralateral brainstem nuclei, we propose that bilateral increase in brainstem respiratory activity contributes to the maintenance of ventilation and recovery of phrenic motor output post-C2Hx. Future work should also assess whether respiratory brainstem activity is progressively altered over time post-injury, and what role this supraspinal plasticity plays in recovery of phrenic motor function.

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Highlights

- Phrenic motor output recovers acutely following incomplete C2Hx
- Neuroplastic reorganization of respiratory related activity occurs acutely within the supraspinal respiratory network after SCI
- Reticular neurons exhibit phasic respiratory-related activity that is increased following cervical SCI

Figure 1. Recovery of phrenic activity post-C2Hx

A. The schematic diagram illustrates connectivity between ventral respiratory group (VRC) and spinal phrenic motoneurons following C2Hx. Blue dot represents phrenic interneurons. Phrenic motor output was determined by electrophysiological recording from the phrenic nerves (PhN): ipsi- PhN, contra- PhN before, 10 min, 1, 2 and 6 hours post C2Hx. B. Ipsilateral and contralateral phrenic nerve recovery as a percentage of pre-injury level at 10 min, 2, 4 and 6 hours post C2Hx. C. Reconstruction of cross-sections through the lesion epicenter shows that not all injuries were complete hemisections.

Figure 2. Examples of respiratory units recorded in the caudal medulla

A. Examples of inspiratory units; B. Examples of expiratory units; PhN – phrenic nerve activity; Unit – single cell activity.

Figure 3. 2D maps of medullary respiratory activity pre- and post-C2Hx

Schematic diagrams of the dorsal surface of the medulla (**A**) and transverse sections (**B**; 40 micron through the medulla, stained with cresyl violet), highlighting (blue shading) the region that recordings were made. Sections from the medulla were taken from naïve animals not included in this study, used here to highlight morphology and nuclei only. Double headed arrows (**B**) estimate 1mm penetration depth from the dorsal surface of brainstem, and 1mm lateral to midline, based on an estimated 60% tissue shrinkage with paraformaldehyde fixation. Stereotaxic coordinates (200 micron apart) used for mapping were measured from calamus scriptorius (CS; 'zero' point), as indicated on photographs of the dorsal surface of the medulla with grid overlay (200 micron squares). Recordings were made 1–2.4 mm from midline and 1 to –1.8 mm rostral and caudal from CS, and 1 – 3.6 mm from the dorsal surface. 2D contour maps have been plotted for uninjured (Control) and C2Hx (Post-C2Hx) animals and overlay with the medulla photograph. These maps represent the proportion of sites that showed expiratory (E, red), inspiratory (I, green) and no respiratory (NRA, blue) activity. The pattern of brainstem respiratory activity is altered within hours post-C2Hx as indicated by contour maps (**C**) and quantified inspiratory and expiratory sites (**D**) rostral and caudal to CS. Graphs in **D** represent the proportion of I (green), E (red) and NRA (blue) within recorded sites above and below calamus scriptorius (SC). Open and filled bars represent control and injured animals, respectively.

The following abbreviations are used: CS – calamus scriptorius; pFRG – parafacial respiratory group; RTN – retrotrapezoid nucleus; Bot – botzinger complex; pre-Bot – pre-botzinger complex; NA – nucleus ambiguus; rVRG- rostral ventral respiratory group; LRN –

lateral reticular nucleus; LPGi- paragigantocellular nucleus; cVRG – caudal ventral respiratory group.; Ra – raphe nucleus; Gi – gigantocellular nucleus. Scale bar is 1mm in B.

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Figure 4. 3D map of medullary respiratory activity pre- and post-C2Hx

Diagrams show series of 2D maps taken from 1 to 3.6mm depths in the caudal medulla, to offer a 3D impression of recorded activity. The distribution of inspiratory and expiratory activity is indicated in green and red, respectively. A – uninjured (Control) animals; B – C2Hx (Post-C2Hx) animals. Note the change in the distribution of recording respiratory activity acutely following C2Hx, and the increase in respiratory-phase activity in the ventral most regions of the brainstem – within the reticular nuclei.