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

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ABSTRACT

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

Introduction

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

The most extensively documented example of respiratory dysfunction and neuroplasticity post-SCI to date is the so-called "crossed-phrenic phenomenon" (CPP) (Goshgarian, 2003, 2009; Lane et al., 2008a, 2009; Zimmer et al., 2007) in which a C_2 lateral hemisection (C_2Hx) results in complete paralysis of the ipsilateral hemidiaphragm. Partial functional recovery can then be induced within hours (Goshgarian, 1979; O'Hara and Goshgarian, 1991) or occur spontaneously weeks after injury (Fuller et al., 2003, 2006, 2008; Golder and Mitchell, 2005; Lee et

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

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al., 2010; Nantwi et al., 1999; Vinit et al., 2007). Both the C_2Hx and more recent C_2 lateralized (hemi-) contusion (Baussart et al., 2006) SCI models are predominantly upper motoneuron (i.e., white matter) injuries since the more caudal PhMN pools are completely spared. While providing important proofs-of-concept for neuroplasticity and repair, such injuries are clinically infrequent (NSCISC, 2008) and do not identify with the more common upper and lower motoneuron pathology associated with contusion/compression trauma at the level of the PhMN pool (Lane, 2011; Lane et al., 2008a).

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

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

Methods

Mid-cervical spinal cord contusions

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

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

Table 1
Details on the average (±SD) resulting injury, ventilatory requirements post-injury, and proportion of animals that could be weaned from ventilation, following contusion with an intended force of either 150 or 250 kilodynes (KD).

Intended impact force	150 KD (n = 28)	250 KD (n = 20)
Actual impact force	154 (±2) KD	229 (±28)
Probe displacement	1296 (±193) μm	1578 (±136) μm
Probe velocity	122 (±4) mm/s	124 (±3) mm/s
Overall% inclusion	70%	35%

Blood oxygen saturation

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

Analysis of ventilation

As previously described (Fuller et al., 2006, 2008), measures of ventilatory behavior were obtained by whole-body barometric plethysmography (Buxco Inc, Wilmington, NC) from awake, unrestrained animals prior to the spinal contusion procedure and then at weekly intervals for 10 weeks post-injury (n = 5 at 150 KD and n = 4 at 250 KD). In addition, a second subset of 150 KD animals (n = 4) was generated and data obtained pre-injury, and at 1 and 10 weeks post-injury, were compared. Rats were exposed to compressed normoxic gas (21% O_2, balance N_2; flow rate = 2 L/min) for an hour of acclimation in the plethysmography chamber, and then exposed to pressurized normoxic, hypercapnic gas mixtures (7% CO_2, 21% O_2, balance N_2; flow rate = 2 L/min). The chamber pressure, room temperature, animal body temperature, and barometric pressure were used in the Drorbaugh and Fenn equation to provide a breath-by-breath display of ventilation (Drorbaugh and Fenn, 1955). Parameters which were derived from the airflow traces included inspiratory tidal volume (V_T, ml/100 g), respiratory frequency (*f*, breaths/min), minute ventilation ($\dot{V}E$, ml/min/100 g), peak inspiratory flow (ml/s), and peak expiratory flow (ml/s). Measurements of V_T and $\dot{V}E$ were expressed relative to body weight (Fuller et al., 2008). Following baseline recordings, animals were subjected to a ten minute period of hypercapnic gas (normoxic, hypercapnic (7% CO_2, 21% O_2, balance N_2) inspired air). The purpose of the hypercapnic challenge was to test the ability of the rats to increase ventilation upon demand. This procedure can reveal functional deficits that are not evident during periods of eupnea or "quiet breathing" (Fuller et al., 2005). Data were averaged from the last ten minutes during baseline and last five minutes during hypercapnic challenge.

Neurophysiology

Terminal bilateral diaEMG recordings were made immediately (n = 5), and 1 (n = 6) and 12 (n = 4) weeks post-contusion just prior to being euthanized and perfusion-fixation for histological analyses (see below). Animals were anesthetized, as described above, and an incision made along linea alba to expose the abdominal