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Respiratory recovery following high cervical hemisection

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Abstract

In this paper we review respiratory recovery following C2 spinal cord hemisection (C2HS) and introduce evidence for ipsilateral (IL) and contralateral (CL) phrenic motor neuron (PhrMN) synchrony post-C2HS. Rats have rapid, shallow breathing after C2HS but ventilation ($\dot{V}_E$) is maintained. $\dot{V}_E$ deficits occur during hypercapnic challenge reflecting reduced tidal volume (V_T), but modest recovery occurs by 12 wks post-injury. IL PhrMN activity recovers in a time-dependent manner after C2HS, and neuroanatomical evidence suggests that this may involve both mono- and polysynaptic pathways. Accordingly, we used cross-correlation to examine IL and CL PhrMN synchrony after C2HS. Uninjured rats showed correlogram peaks consistent with synchronous activity and common synaptic input. Correlogram peaks were absent at 2 wks post-C2HS, but by 12 wks 50% of rats showed peaks occurring with a 1.1 ± 0.19 ms lag from zero on the abscissa. These data are consistent with prolonged conduction time to IL (vs. CL) PhrMNs and the possibility of polysynaptic inputs to IL PhrMNs after chronic C2HS.

Keywords

spinal cord injury; breathing; phrenic; plasticity

1. Introduction

Hemisection from the midline to lateral edge of the cervical spinal cord has been used extensively to study respiratory plasticity following spinal cord injury (SCI) (Goshgarian 2003; Fuller et al. 2005b; Lane et al. 2008a). The basic premise is that C2 hemisection (C2HS) interrupts descending bulbospinal pathways from the medulla to phrenic motoneurons (PhrMNs) located ipsilateral (IL) to the lesion. Thus, the IL hemidiaphragm is transiently

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paralyzed but contralateral (CL) diaphragm activity persists. Compensation via activation of CL PhrMNs and other respiratory pathways is sufficient to maintain minute ventilation ($\dot{V}_E$) thereby enabling the animal to survive the lesion. Subsequently, recovery processes affecting respiratory motor output can be studied. The purpose of this article is two-fold. First, we will review and summarize current knowledge regarding respiratory recovery and compensation following C2HS. Second, we will present original neurophysiological data from our laboratory which provides insight into the potential neural substrate for recovery of IL phrenic activity (i.e. the crossed phrenic phenomenon or CPP) following chronic C2HS.

2. The C2HS model and midline tissue sparing

Prior to discussing the functional impact of C2HS on breathing, a brief discussion regarding the “anatomical completeness” of cervical hemilesion injury is warranted. Confusion arises regarding the following question: for demonstration of the CPP, does a hemilesion need to extend to the spinal midline, or can some ventromedially (VM) located white matter be spared? Perhaps the most critical observation is that a portion of the bulbospinal axons innervating IL PhrMNs are found in the VM cervical white matter (Lipski et al. 1994; Fuller et al. 2009). Accordingly, one might predict that cervical hemilesions which spare the VM spinal cord will also fail to abolish inspiratory bursting in IL PhrMNs. This prediction is supported by the data of Li and Decherchi (2003), but others have reported that partial cervical hemilesions abolish (at least transiently) IL phrenic output (Goshgarian 1981; Vinit et al. 2007). We recently compared phrenic motor output and $\dot{V}_E$ between C2 hemilesioned rats with a small degree of VM white matter sparing vs. rats with anatomically complete C2HS (Fuller et al. 2009) (see Fig. 1). The group with spared VM pathways showed greater tidal volume (V_T) and more robust IL phrenic bursting during chemical respiratory challenge. Moreover, the incompletely lesioned rats had lower breathing frequency (fR) during both unanesthetized and anesthetized conditions. Accordingly, we suggest that medially located fibers can impact respiratory outcomes after cervical hemilesion (Fuller et al. 2009). Although lateral hemilesion with VM tissue sparing is an important model with some key experimental advantages (Kastner et al. 2008), in this review we will emphasize studies with anatomically or physiologically confirmed C2HS lesions.

3. Recovery of respiratory motor function after C2HS

3.1. Recovery, plasticity and compensation

Cervical SCI always alters breathing, and both humans as well as experimental animals often show some degree of respiratory recovery following chronic cervical SCI (Nantwi et al. 1999b; Winslow et al. 2003; Fuller et al. 2008). However, respiratory recovery in mice may be blunted relative to other species (see Seeds et al., this volume). For this report we define recovery as the return of $\dot{V}_E$ or other indices of respiratory function (e.g. vital capacity, nerve activity, etc.) towards values that would be expected in spinal-intact individuals of comparable age, sex and body mass. The recovery process can occur through a range of mechanisms, many of which represent types of *neuroplasticity*. A working definition was put forth by Mitchell and Johnson (2003) who defined respiratory-related neuroplasticity as “a persistent change in the neural control system (morphology and/or function) based on prior experience”. Within the context of this definition, it is useful to differentiate between *compensation* (or compensatory plasticity) in neurologically intact pathways vs. *plasticity* in neurons and/or networks directly impaired by C2HS (e.g. denervated IL PhrMNs; see also Lane et al. this volume). While both these processes fit under the broad definition of plasticity (i.e. a “persistent change”, Mitchell and Johnson 2003), the underlying mechanisms are likely much different. Compensation after SCI, for example, involves increased recruitment of other respiratory muscles via intact neural pathways, and may reflect persistently altered sensory feedback (Teitelbaum et al. 1993; Katagiri et al. 1994; Brichant et al. 1997). On the other hand, changes

in synaptic connections or other adaptations that increase the efficacy of neurotransmission to IL PhrMNs are examples of plasticity in a neurologically impaired pathway. Thus, the terms plasticity and compensation, while not mutually exclusive, generally are used to describe mechanistically different processes. In this manuscript we will use the term compensation to describe alterations in CL respiratory output and/or accessory muscle recruitment after C2HS.

3.2. Ventilation after C2HS

If the C2HS model is to be used to test potential therapeutic interventions (e.g. theophylline (Nantwi et al. 1996), cAMP (Kajana et al. 2008), hypoxia (Golder et al. 2005), light-activated proteins (Alilain et al. 2008b)), then it is important to determine how this injury impacts $\dot{V}_E$ and related behaviors such as augmented breaths. An important caveat is that while $\dot{V}_E$ measures can describe the recovery of breathing, such measures will reveal little about the mechanism of recovery (e.g. plasticity and compensation). Nevertheless, behavioral measures such as $\dot{V}_E$ provide an important complement to neuroanatomical, neurophysiological, and molecular studies of respiratory plasticity after SCI.

To date, several studies have examined the impact of C2HS on $\dot{V}_E$ (Goshgarian et al. 1986; Nantwi et al. 1999a; Golder et al. 2001b; Golder et al. 2003; Fuller et al. 2006; Fuller et al. 2008). The initial report was from Goshgarian and colleagues (1986) who demonstrated that female rats breathe with an elevated frequency at approximately 24 hr post-C2HS. Arterial blood gases were consistent with hyperventilation as reflected by increased arterial pO_2 and a tendency for decreased arterial pCO_2 (Goshgarian et al. 1986). It was subsequently reported that rats transiently hypoventilate for a few hours after C2HS (Fuller et al. 2005b) but by 2-wks post-injury their arterial blood gases are not different than uninjured control rats (Miyata et al. 1995). Golder et al. (2001b) used pneumotachography to study the pattern of breathing in anesthetized, tracheotomized C2HS female rats breathing room air. Relative to uninjured control animals, C2HS rats had increased breathing frequency (fB) and reduced $\dot{V}_T$ at both 1 and 2 months post-injury. Further, bilateral vagotomy caused the rapid, shallow breathing pattern to return to control values thereby demonstrating that vagal mechanisms contribute to the pattern of breathing after C2HS. Golder et al. (2001b) also showed that sighs or augmented breaths occur more frequently after C2HS. Augmented breaths prevent lung atelectasis and are impaired in human SCI (McKinley et al. 1969). Thus, the augmented breath is a potentially useful outcome measure in pre-clinical rodent SCI models (see Bolser et al., this volume).

The first investigation of ventilation in unanesthetized, unrestrained rats after chronic C2HS was conducted by Fuller et al. (2006) using barometric plethysmography (see Mortola & Frappell 1998 for review and commentary on the method). In that study, $\dot{V}_E$ was examined in male rats during a baseline period (21% O_2) and a hypercapnic challenge (21% O_2 , 7% CO_2) at 2–5 wks post-injury. The respiratory challenge is particularly important because chemical stimulation of breathing (i.e. hypoxia, hypercapnia) can activate IL phrenic pathways after C2HS (see below). Rats maintained $\dot{V}_E$ with a rapid, shallow breathing pattern (reduced $\dot{V}_T$, increased fB) that persisted through the duration of the study. Deficits in $\dot{V}_E$ were revealed during the hypercapnic challenge and reflected reduced $\dot{V}_T$. There was no evidence for recovery of $\dot{V}_T$ or $\dot{V}_E$ over the 5 wk post-injury period. However, in that study $\dot{V}_T$ or $\dot{V}_E$ were compared to pre-injury measurements in the same rats (i.e. repeated measures). This approach may not adequately control for differences in body mass or age between the injured and spinal intact conditions. Further, Nantwi et al. (1999a) showed progressive improvements in diaphragm EMG activity over intervals > 5 wks post-C2HS suggesting that $\dot{V}_E$ recovery might be more robust at later time points. Accordingly, we recently examined $\dot{V}_E$ up to 3 mos post-C2HS in male rats and compared the data to age, weight and sex matched controls (Fuller et al. 2008). Similar to prior reports (Fuller et al. 2005b; Fuller et al. 2006), at 2 wks post-C2HS $\dot{V}_E$ was maintained during baseline conditions (21% O_2) but was substantially blunted during

hypercapnic challenge (68% of $\dot{V}_E$ in uninjured, weight-matched rats). However, by 3 mos the injured rats achieved a hypercapnic $\dot{V}_E$ that was 85% of control. Thus, the ability to increase $\dot{V}_E$ during respiratory challenge showed a modest but significant recovery by 3 mos post-C2HS. C2HS rats also exhibited augmented breaths with reduced volume and greater frequency than controls (Fuller et al. 2008). AB volume tended to be greater at 3 mos (vs. 2 wks) post-injury but remained well below values observed in control rats. Thus, some degree of $\dot{V}_E$ recovery occurs after C2HS, but endogenous neuromuscular plasticity and/or compensation appears to be insufficient to promote full respiratory recovery.

Recovery of $\dot{V}_E$ following SCI may be different between males and females (Doperalski et al. 2008). Indeed, there are numerous reports of improved functional and/or histological outcomes in female vs. male rodents following central nervous system injury (reviewed in Roof et al. 2000) and exogenous estrogen therapy can improve motor recovery after SCI (Chaovipoch et al. 2006). Sex hormones can act as respiratory stimulants and also modulate the expression of plasticity in respiratory motoneurons (Behan et al. 2008). Accordingly, we recently compared respiratory recovery between male and female rats following C2HS (Doperalski et al. 2008). Significant differences in the pattern of breathing were seen between sexes following C2HS, although assessments were only completed at two wks post-injury. In particular, post-injury reductions in $\dot{V}_T$ observed during hypercapnic challenge were significantly more pronounced in males vs. females. This gender difference was reduced considerably when females were ovariectomized prior to C2HS suggesting that it may have been mediated by ovarian sex hormones.

3.3. Recovery of IL phrenic neurogram and diaphragm electromyogram (EMG) activity following C2HS

The amplitude and post-injury onset time of IL phrenic inspiratory bursting after C2HS (i.e. the CPP) is variable across published reports. However, direct comparisons across previous studies are confounded by potential variability in genetics (Fuller et al. 2001), sex (Doperalski et al. 2008), C2HS methods, and neurophysiology recording conditions and protocols. For example, the expression of respiratory plasticity differs between substrains of Sprague-Dawley rats (Fuller et al. 2001), and the CPP may be more robust in female vs. male Sprague-Dawley rats at 2 wks post-C2HS (Doperalski et al. 2008). Despite variability introduced by these (and other) factors, common themes have emerged from the C2HS literature. In the remainder of this section, we review the spontaneous appearance of the IL phrenic and diaphragm activity after C2HS (i.e. the spontaneous CPP; Fuller et al. 2008).

IL phrenic inspiratory motor output is absent during spontaneous breathing within hours-days post-C2HS, a finding that has been consistent across laboratories and experimental preparations. However, during this acute post-injury period, phrenic bursting can be evoked by sectioning the CL phrenic nerve (Porter 1895; Goshgarian 1981) and also by pharmacologic treatments that increase respiratory drive (Nantwi et al. 1998) or serotonergic function (Zhou et al. 1999; Fuller et al. 2003). Thus, a neural circuit capable of activating IL PhrMNs is present, but is not normally functional acutely following C2HS.

The circuitry underlying the CPP appears to be influenced by segmental inputs including phrenic afferent neurons (Goshgarian 1981). The original demonstration by Porter (1895) showed that CL phrenicotomy caused the rapid appearance of IL hemidiaphragm movements in both dogs and rabbits. In addition to axotomizing phrenic motoneurons, phrenicotomy severs the afferent neurons which comprise a substantial portion of the phrenic nerve (Road 1990). To more selectively examine the impact of cervical afferent neurons, Goshgarian (1981) examined the impact of cutting the cervical dorsal roots on the CPP. He found that CL cervical dorsal rhizotomy resulted in the appearance of IL diaphragm inspiratory EMG activity in rats at 3–28 days post-injury. Similarly, Vinit et al. (2007) showed that CL phrenicotomy caused

an abrupt increase in IL phrenic bursting in rats with lateral cervical lesion. These data support the concept that CL afferent neurons projecting to the cervical spinal cord can inhibit IL phrenic motor output after SCI (Goshgarian 1981). It must be noted, however, that the investigations by Vinit (2007) and Goshgarian (1981) were done using rats with anatomically incomplete hemilesion as demonstrated by the published histology. Midline tissue sparing has a significant impact on phrenic motor output after cervical hemilesion (Fuller et al. 2009), and thus the potential impact of activating phrenic and other cervical afferent neurons on the CPP could be influenced by the sparing of ventromedially located white and gray matter.

There is also evidence that the integrity of the IL phrenic nerve has a strong influence on the expression of the CPP. Golder et al. (2003) observed that IL phrenic bursting was always absent in rats two months following a dual injury consisting of C2HS and IL phrenicotomy (distal to the subsequent recording site). In contrast, 80% of C2HS rats with an intact IL phrenic nerve showed IL inspiratory bursting under comparable conditions. Similarly, Vinit and colleagues (2007) demonstrated that IL phrenicotomy distal to the recording site immediately abolishes IL phrenic inspiratory bursting in rats with lateral cervical SCI. Accordingly, expression of the CPP may be facilitated by activation of IL phrenic afferents.

There is agreement in the literature that IL PhrMN output increases spontaneously over a period of wks-mos post-C2HS in rats. This response could reflect plasticity in and around PhrMNs (Goshgarian et al. 1989; Mantilla et al. 2003) and/or the increases in the synaptic efficacy of bulbospinal or propriospinal inputs (Lane et al. 2008a). In either case, the result is that IL phrenic bursting, which initially is absent after C2HS, returns during “quiet breathing”. This was first reported by Nantwi and colleagues (Nantwi et al. 1999a) and has since been confirmed by multiple laboratories. However, the duration of time which precedes the appearance of IL PhrMN bursting varies markedly between studies. To our knowledge, the earliest that spontaneous crossed-phrenic inspiratory activity has been demonstrated in rats with histologically confirmed C2HS lesions is 2 wks post-injury (Golder et al. 2005; Fuller et al. 2006; Fuller et al. 2008; Fuller et al. 2009). In most of these experiments, rats were paralyzed, ventilated and vagotomized and recordings were made from the proximal cut end of the phrenic nerve. In contrast, anesthetized and spontaneously breathing vagally-intact rats show no diaphragm EMG evidence of crossed phrenic activity at 2 wks post-injury (Mantilla et al. 2007). Similarly, Nantwi et al. (1999b) showed that IL phrenic activity is absent during normoxic breathing in vagally-intact rats at 4 weeks post-C2HS but becomes relatively robust by 10–16 weeks. The discrepancy in CPP onset between vagal-intact vs. vagotomized preparations suggests that IL phrenic output after C2HS may be particularly susceptible to inhibition via vagal afferent neurons (Chatfield et al. 1948). Consistent with this notion, we have recently observed that vagal afferents have a more robust inhibitory influence on IL as compared to CL phrenic bursting at 2 wks post-C2HS (K-Z Lee and DD Fuller, unpublished observations).

3.4. Mechanisms of IL PhrMN recovery

Several groups have explored morphological and molecular plasticity in the brain (Golder et al. 2001a; Zimmer et al. 2007b) and spinal cord (Goshgarian 2003; Mantilla et al. 2003; Lane et al. 2008b; Zimmer et al. 2008) after C2HS. For example, Goshgarian and colleagues have described synaptic remodeling in the IL spinal cord occurring within hours of C2HS (Sperry et al. 1993). Mantilla et al. (2003) have reported decreases in PhrMN size at 2 wks post-C2HS. Molecules that may contribute to recovery of phrenic output after C2HS, at least under certain circumstances, include serotonin (Hadley et al. 1999; Fuller et al. 2005a), cAMP (Kajana et al. 2008), adenosine (Nantwi et al. 2005), neurotrophins including BDNF (Seick et al. 2008), glutamate (Alilain et al. 2008a), and GABA (Zimmer et al. 2007a). Other papers in this issue of the Journal are addressing these mechanisms and they are not reviewed in here.

3.5. Does the CPP contribute to recovery of $\dot{V}_E$ after C2HS?

The CPP provides an excellent experimental model to examine the mechanisms associated with spontaneous or induced recovery of electrical activity in paralyzed muscles. It is not necessarily certain, however, that the CPP will substantially impact $\dot{V}_E$ in awake animals. For example, a functional impact on respiratory pressures and volumes during breathing may not be detectable if a relatively small fraction of IL PhrMNs are active during the CPP. Further, compensation from CL pathways can be robust after C2HS (see below) and may be sufficient to maintain $\dot{V}_E$ without contribution from the CPP (Fuller et al. 2006). However, Golder et al. (2003) demonstrated that the CPP makes a measurable contribution to breathing after chronic C2HS in anesthetized rats. In their study, female rats with both C2HS and IL phrenicotomy (i.e. a dual injury preventing crossed phrenic impulses from reaching the diaphragm) had V_T 's that tended to be less than rats with C2HS only (5.6 ± 0.2 vs. 6.1 ± 0.1 ml/kg). More importantly, the volumes of spontaneous augmented breaths were significantly lower in rats with the dual injury (~ 4 ml/kg) vs. C2HS alone (~ 11 ml/kg) (see Fig. 2 in Golder et al. 2003). Augmented breaths are associated with very high respiratory drive and activation of a much larger number of PhrMNs as compared to eupnea or chemoreceptor stimulation (Sieck et al. 1989). We recently observed that V_T in unanesthetized male rats shows progressive recovery after C2HS. Improvements in V_T over 2–12 wks post-C2HS were observed during both baseline (quiet breathing, 21% O_2) and during a hypercapnic challenge (Fuller et al. 2008). Accordingly, we suggest that the spontaneous CPP makes a functional contribution to improved breathing after chronic C2HS. The contributions of the CPP may be more apparent under conditions of elevated respiratory drive (e.g. exercise, augmented breaths, etc.) but also may be relevant during conditions of eupneic breathing after chronic injury.

3.6.1. Compensation in CL hemidiaphragm output after C2HS—Studies of respiratory plasticity and recovery after C2HS have logically focused on IL phrenic and diaphragm activity as the intent has been to study the CPP (Goshgarian 2003). However, C2HS also offers the opportunity to study compensatory responses in other respiratory pathways. CL diaphragm EMG amplitude increases by approximately 50% following C2HS in rats (Miyata et al. 1995; Rowley et al. 2005), and a similar response has been described following diaphragm hemiparesis in dogs (Teitelbaum et al. 1993; Katagiri et al. 1994). This increase in CL hemidiaphragm activity has been suggested to represent the removal of an inhibitory input mediated by large diameter phrenic afferents from tendon organs, or possibly activation of small diameter phrenic afferents (type III and IV) (Teitelbaum et al., 1993). However, CL diaphragm compensation does not appear to reflect vagal mechanisms, at least in dogs (Teitelbaum et al. 1993). Interestingly, CL respiratory motor output may actually be inhibited under certain conditions following C2HS. Golder et al. (2001a) reported diminished CL phrenic burst amplitude during a respiratory hypercapnic challenge in vagotomized and ventilated rats studied 2 mos post-C2HS. This response was attributed to a supraspinal mechanism because hypoglossal nerve activity was similarly reduced. However, the diminished CL phrenic response was not observed in rats which did not spontaneously express the CPP. Thus, CL phrenic motor plasticity may be influenced by the presence (or absence) of crossed phrenic activity.

3.6.2. Compensation in other respiratory muscles after SCI—It is well established that diaphragm paralysis induces compensatory increases in the activity of other respiratory muscles in species ranging from rats to humans (Sherrey et al. 1990; Brichant et al. 1997; Winslow et al. 2003). For example, bilateral phrenicotomy in rats induces a robust increase in intercostal muscle EMG activity (Sherrey et al. 1990). It is worth noting that rats not only survive bilateral phrenicotomy, but this procedure has only a minor impact on sleep-waking patterns (Sherrey et al. 1990). Similarly, dogs have only minor reductions (Katagiri et al., 1994) or even no change in V_T (Stradling et al. 1987) following bilateral phrenicotomy. The

relative contribution of other respiratory muscles to the compensatory $\dot{V}_E$ response has not been evaluated in the rat C2HS model. However, several studies have described compensation following unilateral diaphragm paralysis in dogs (Teitelbaum et al. 1993; Katagiri et al. 1994). Teitelbaum et al. (1993) showed that hemidiaphragm paresis causes a robust increase in CL parasternal and alae nasi EMG activity. Similarly, Katagiri et al. (1994) demonstrated that unilateral diaphragm paralysis induces robust compensatory increases in parasternal and transverse abdominus EMG activity in anesthetized dogs. This response appears to result from the removal of inhibitory inputs mediated through phrenic afferent neurons (probably originating from diaphragm tendon organs) with some respiratory stimulation reflecting arterial hypercapnia (Teitelbaum et al. 1993; Brichant and DeTroyer 1997). Since rats do not show persistent hypercapnia following C2HS (Goshgarian et al. 1986; Miyata et al. 1995), it is likely that any compensatory responses after this injury are mediated by afferent signals from the diaphragm (Teitelbaum et al. 1993; Brichant and DeTroyer 1997), intercostals, abdominal muscles and lungs (Golder et al. 2001b).

4. Cross-correlation and phrenic motoneuron synchrony

4.1. Descending control of PhrMNs: mono- vs. polysynaptic circuits

The appearance of IL PhrMN inspiratory activity following both acute (i.e. min to days post-injury) and chronic C2HS has been attributed to activation of a monosynaptic, bulbospinal pathway which crosses the spinal midline caudal to the injury (Goshgarian 2003). Goshgarian and colleagues have provided clear anatomical evidence for the existence of such “crossed phrenic pathways” (Goshgarian 2003). However, recent neuroanatomical data from our group suggests that the descending control of PhrMNs in the rat may also involve propriospinal cervical interneurons (see Lane et al. this volume; Lane et al. 2008a, 2008b). There is neurophysiological evidence that some PhrMNs may be activated by descending, polysynaptic pathways in spinal intact rats (Duffin et al. 1995; Tian et al. 1996). Similarly, neurophysiological evidence of polysynaptic inputs to PhrMNs in C2HS rats was provided by Ling et al. (1995). Specifically, electrical stimulation of the ventral funiculus in the CL spinal cord evoked compound potentials in the IL phrenic nerve with both short (i.e. ~ 1.0 ms) and relatively long onset latencies (i.e. 5–7 ms). The long latency peaks are consistent with polysynaptic inputs in IL PhrMNs (Ling et al., 1995). While definitive evidence of a role for cervical interneurons in respiratory recovery after SCI is lacking, there is strong evidence in other motor pathways (e.g. corticospinal) that cervical interneurons can promote functional recovery (Bareyre et al. 2004). We reasoned that if time-dependent recovery of IL PhrMN activity following chronic C2HS involves activation of propriospinal cervical interneurons, then measurements of synchrony between IL and CL PhrMN discharges might reveal some features of the circuitry involved. Thus, we used cross-correlation analyses (Kirkwood 1979) to examine the synchrony of bursting between IL and CL PhrMNs after chronic C2HS. We provide a brief overview of this method (section 4.2) followed by a summary of our results (section 4.3).

4.2. Cross-correlation as a tool to examine PhrMN synchrony

Detailed critiques and descriptions of the relevant methodologies have been published (Kirkwood 1979; Hamm et al. 2001; Peever et al. 2001). Cross-correlation, as used in the present analysis is employed to examine the temporal relationship between two neural signals. In the context of C2HS, the relevant signals are the IL and CL phrenic neurograms. Using CL output as the reference signal, spikes recorded in the IL neurogram are correlated with CL spikes. This analysis yields a cross-correlation histogram (correlogram) which presents the spike occurrences in the IL neurogram (ordinate) relative to the time of occurrence of spikes in the CL neurogram (Kirkwood 1979) (abscissa; Fig. 2). The presence of a peak in the resulting correlogram indicates that the probability of IL and CL spikes occurring simultaneously or

sequentially (i.e. time-locked, but delayed) is greater than should occur by chance (Kirkwood 1979). A correlogram peak which is centered at zero on the ordinate (Fig. 2) indicates a high probability of simultaneous bursting (spikes) from IL and CL PhrMNs, and a relatively narrow peak suggests a common, monosynaptic input to these cell populations (Duffin et al. 1995). For instance, a single ventral respiratory column (VRC) inspiratory neuron which anatomically branches to innervate PhrMNs on both the IL and CL side of the spinal cord could produce just such a correlogram peak (Duffin et al. 1995). In contrast, a correlogram peak that is offset (i.e. shifted to the right of zero on the ordinate) is consistent with delayed activation of one cell population (i.e. IL vs. CL PhrMNs). In addition, Kirkwood and colleagues (Vaughan et al. 1997) suggest that relatively broad correlogram peaks are consistent with actions of common di- or oligosynaptic inputs during breathing.

4.3. Cross-correlation of IL and CL phrenic neurograms following C2HS

4.3.1. Methods—Adult male Sprague-Dawley rats were studied following approval by the Institutional of Animal Care and Use Committee at the University of Florida. The C2HS lesion (left side) and post-mortem histological assessment have been described (Fuller et al. 2009). Bilateral phrenic neurograms were recorded (sample rate 10,000 Hz) in urethane-anesthetized, paralyzed, vagotomized and ventilated rats (Fuller et al. 2009). Electrical activity was amplified (1000x), band-pass (300–10,000 Hz) and notch (60 Hz) filtered as described previously (Fuller et al. 2008). The end-tidal CO₂ was maintained 10–15 mmHg above the apneic threshold during all recordings to enable a robust signal-to-noise ratio in the IL phrenic neurogram. The sensitivity of the cross-correlation method improves with longer recording durations (Kirkwood 1979). Neurograms were thus recorded for approximately 3 hours (mean = 174±14 min) resulting in an average of 302,573±66,026 events per correlogram. The raw phrenic signal was converted into events by setting a threshold just above the noise level in the phrenic neurograms (see Fig. 2). The left (IL) event train was then cross-correlated with the right (CL) reference train using 0.2-ms bin widths using Spike2 software (Cambridge Electronic Design Limited, Cambridge, England). Correlogram baseline and peaks were discerned using the method described by (Davies et al. 1985). Data are presented as the mean ± standard error.

4.3.2. Results—All C2HS lesions were confirmed histologically to extend to the spinal midline (e.g. Fig. 1A). All animals displayed rhythmic inspiratory bursting in both IL and CL phrenic nerves (Fig. 2A). Correlograms computed from IL and CL phrenic neurograms in uninjured rats (n=5) displayed peaks with a mean half-width of 1.08±0.14 ms (Fig. 2Ci, Cii). Correlogram peaks occurred at zero in 3 rats but showed a slight positive lag in the remaining 2 rats (0.1 and 0.6 ms). No discernable peaks were observed in correlograms from 2 wk post-C2HS rats (n=5) (Fig. 2Ciii, Civ), despite comparable baseline counts in the histograms (and hence sensitivity for detection of peaks). In contrast, correlogram peaks were observed in 6 of 12 rats studied at 12 weeks post-C2HS. The half-widths of these peaks were similar to values in uninjured rats (1.05±0.11 ms). However, correlogram peaks observed at 12 wks post-C2HS were shifted away from zero by the following lags, 0.3, 0.9, 1.1, 1.3, 1.3, 1.7 (mean = 1.1±0.19; p=0.003 vs. uninjured, Mann-Whitney test). Five of these were shifted to the right (e.g. Fig. 2Cv), but one was shifted to the left (lag 0.9, Fig. 2Cvi). Moreover the example with the smallest shift, was a peak which had a k-value (Sears et al. 1976) of 1.77, and therefore demonstrated synchrony which was at least 10 times stronger than any of the normal examples (k between 1.009 and 1.068) or the other C2HS animals (k between 1.015 and 1.060), as illustrated in Fig. 2C. The ET-CO₂ apneic threshold, arterial blood gases, and mean arterial pressure were all similar between groups (all p>0.05; data not shown).

4.3.3. Discussion—Cross-correlation of phrenic signals in spinal intact rats revealed correlogram peaks centered at zero as previously reported (Duffin et al. 1995; Peever et al. 2001). The presence of these peaks indicates that a subset of IL and CL PhrMNs receive a

common synaptic input (note: IL and CL are arbitrary terms in spinal intact rats). A common input could, in theory, reflect brainstem synchrony (e.g. precise synchronization of IL and CL medullary premotor neurons) or simultaneous excitation of IL and CL PhrMNs by shared premotor neurons. This latter possibility could reflect simultaneous IL and CL PhrMN activation via collaterals that cross the spinal midline (Moreno et al. 1992). Bilateral PhrMN synchrony probably does not reflect activation of dendrites which cross the midline because such processes are rare in adult rats (Prakash et al. 2000). The most likely explanation for IL and CL PhrMN synchrony in spinal intact rats is activation of medullary premotor neurons with two descending projections: one which crosses the midline in the medulla and another which descends caudally without crossing (Duffin et al. 2006).

Clear IL phrenic bursting was observed at 2 wks post-C2HS as previously reported (Fuller et al. 2008). However, cross-correlation of IL and CL phrenic neurograms did not yield correlogram peaks. One interpretation of this apparent lack of synchrony is that distinct medullary populations are responsible for activation of PhrMNs on the IL vs. CL sides of the cord. In other words, “crossed phrenic pathways”, as assessed at 2 wks post-C2HS, may innervate only IL PhrMNs with no collateral projection to CL cells. It must be mentioned, however, that precisely equivalent populations of motoneurons may not be sampled at the different time points, either because of different physiological recruitment or because of the somewhat arbitrary levels chosen for event discrimination. Motoneurons of different sizes may not be equally synchronized. In any case, the absence of a correlogram peak at 2 wks post-C2HS does not exclude a potential contribution of cervical interneurons to activation of IL PhrMNs. However, if interneurons are part of the CPP circuit at this time point they are probably not innervated by medullary cells also projecting to CL PhrMNs.

A common synaptic input to IL and CL PhrMNs was detected in 50% of rats at 12 wks post-C2HS as shown by discernable correlogram peaks (Fig. 2). Since correlogram peaks were not observed at 2 wks post-injury, this finding may reflect time-dependent plasticity within the phrenic motor circuitry. For example, sprouting of descending CL fibers across the spinal midline could result in progressive recovery and common innervation of IL and CL PhrMNs. Correlogram peaks present at 12 wks post-injury which showed a lag or rightward shift from zero are consistent with delayed onset of IL PhrMNs. One or more mechanisms could contribute to these effects. First, for the peaks with a rightward shift, slower conducting axons may play a larger role in the transmission of excitatory drive to the IL vs. CL side. Second, IL PhrMNs may be less excitable after C2HS. However, Mantilla and Sieck (2003) reported a reduction in PhrMN soma size after C2HS, a finding which argues against a reduction in overall excitability. Another possibility, however, is that the peak lag reflects a more complex (polysynaptic) circuit (Vaughan et al. 1997) controlling IL PhrMNs in at least a subset of rats with chronic C2HS. In addition, the appearance of a peak to the left of zero indicates delayed activation of PhrMNs on the right (CL) with respect to those on the left (IL), possibly reflecting an input which originates on the left. The input concerned would most likely be non-respiratory, e.g. intrinsic spinal cord interneurons tonically firing. This would also be the most obvious hypothesis for the single example with very strong synchrony (i.e. high k-value). Evidence for the release of tonic interneuron activity acting to support the respiratory phased discharges of inspiratory thoracic motoneurons was provided by Kirkwood et al (1984). Overall, the present results suggest an heterogeneous group of inputs becoming active to support the recovered IL PhrMN activity following C2HS. Such a view is consistent with recent neuroanatomical data (Lane et al. 2008a), but a direct test of this hypothesis must await more detailed analyses of cervical interneuron behaviour, both neuroanatomical and neurophysiological, after C2HS.

5. Summary and conclusion

C2HS induces a persistent alteration in respiratory control as shown by neurophysiological outcomes (e.g. neurogram and EMG studies) and measures of breathing (e.g. plethysmography, pneumotachography) after chronic injury. However, an incomplete but significant respiratory recovery occurs after C2HS. This recovery probably reflects both plasticity in IL phrenic motor output and compensation from CL pathways and other respiratory muscles. Both neurophysiological and neuroanatomical data suggest that the neural circuitry underlying IL PhrMN recovery may be more complex than previously envisioned.

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Figure 1. Representative histological sections of the cervical spinal cord following hemilesion
 Panel A presents both longitudinal and transverse sections of a anatomically complete C2HMx injury. Panel B provides longitudinal and transverse sections of an incomplete C2HMx with ventromedial tissue sparing. The arrow in panel B indicates the area of midline tissue sparing. The longitudinal sections (40 μ m, vibratome) are labeled with antibodies to pseudo-rabies virus (PRV; as part of a separate study); the transverse sections (40 μ m, vibratome) are stained with luxol fast blue and cresyl violet. Scale bars are 1mm.

Figure 2. Examples of phrenic neurograms and correlograms

Panel A presents phrenic neurograms recorded both ipsilateral and contralateral to C2HMx lesion at 2 (middle) and 12 (bottom) wks post-injury. Bilateral phrenic bursting in an uninjured rat is shown in the top traces of Panel A. Time and amplitude scaling are identical in each trace; phrenic signals were amplified 1000x. Panel B shows an expanded time scale of the phrenic burst area indicated by the gray line in panel A. In addition, the event markers generated via spike2 software to enable cross-correlation (see methods) are presented immediately above or below the corresponding neurogram signal. Panel C shows correlograms generated via cross-correlation of phrenic event markers (see methods). The number of counts is shown on the Y-axes. In panel C, the top correlograms (i.e., Ci, Ciii and Cv) were generated from the complete

record (i.e. ~ 3 hr of data) associated with the phrenic data depicted in panel A. The bottom correlograms in panel C (i.e. Cii, Civ, Cvi) are provided as additional representative examples. While correlogram peaks were always observed in uninjured rats (Ci, Cii), they were never observed at 2 wks post-C2HMx (Ciii, Civ) and occurred in 50% of rats (6/12) at 12 wks post-C2HMx (Cv, Cvi). The arrow in panel Cvi indicates the presence of a peak occurring with a negative lag (see results).