Published in final edited form as:

Respir Physiol Neurobiol. 2009 November 30; 169(2): 123–132. doi:10.1016/j.resp.2009.08.007.

Spinal circuitry and respiratory recovery following spinal cord injury

Michael A. Lane¹, Kun-Ze Lee², David D. Fuller², and Paul J. Reier¹

¹ Department of Neuroscience University of Florida College of Medicine and McKnight Brain Institute Rm. L1-100K Box 100244 100 Newell Dr. Gainesville, FL. 32610

² Department of Physical Therapy University of Florida College of Public Health and Health Professions and McKnight Brain Institute Box 100154 100 S. Newell Dr. Gainesville, FL. 32610

Abstract

Numerous studies have demonstrated anatomical and functional neuroplasticity following spinal cord injury. One of the more notable examples is return of ipsilateral phrenic motoneuron and diaphragm activity which can be induced under terminal neurophysiological conditions after high cervical hemisection in the rat. More recently it has been shown that a protracted, spontaneous recovery also occurs in this model. While a candidate neural substrate has been identified for the former, the neuroanatomical basis underlying spontaneous recovery has not been explored. Demonstrations of spinal respiratory interneurons in other species suggest such cells may play a role; however, the presence of interneurons in the adult rat phrenic circuit – the primary animal model of respiratory plasticity – has not been extensively investigated. Emerging neuroanatomical and electrophysiological results raise the possibility of a more complex neural network underlying spontaneous recovery of phrenic function and compensatory respiratory neuroplasticity after C2 hemisection than has been previously considered.

Keywords

Spinal cord injury; plasticity; phrenic; interneuron; neuroanatomical tracing; respiration

1. Introduction

Considerable experimental evidence now exists for post-SCI respiratory neuroplasticity. This potential for intrinsic recovery has been most extensively described in a rat model of high cervical (C2) spinal hemisection (HS) and is often referred to as the crossed-phrenic phenomenon (CPP) (Goshgarian 2003). Initial studies of this injury model showed that recovery of activity in the ipsilateral phrenic nerve and hemidiaphragm can be elicited by contralateral phrenicotomy within minutes to hours after injury. It has since been found that recovery of ipsilateral phrenic nerve and diaphragm activity also can occur spontaneously over the course of weeks (Fuller et al. 2003; Golder et al. 2005; Fuller et al. 2006; Vinit et al.

© 2009 Elsevier B.V. All rights reserved.

Correspondence to: Paul J. Reier, Ph.D. Department of Neuroscience University of Florida College of Medicine and McKnight Brain Institute Box 100244 Rm. L1-100K 100 Newell Dr. Gainesville, FL. 32610 reier@mbi.ufl.edu Phone: 352-392-8696 Fax: 352-392-0439.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

2007; Fuller et al. 2008) to months (Nantwi et al. 1999; Golder et al. 2001a) following C2HS. For present discussion purposes, this delayed natural recovery is operationally referred to as the “spontaneous crossed-phrenic phenomenon (sCPP)”. While the C2HS lesion model (in which both the CPP and sCPP can be expressed) is not typical of the majority of clinical cases of cervical SCI, it provides a valuable experimental setting for demonstrating proof-of-principle pertaining to respiratory recovery (reviewed in Lane et al. 2008a).

Often lacking in SCI research is detailed identification of neural substrates engaged in functional improvements occurring either naturally or in response to therapeutic interventions. Regarding the CPP and sCPP, the neural substrates, may be quite different as suggested by the striking differences in their expression. To date, the CPP has been attributed to activation of a normally silent crossed intraspinal pathway which can restore inspiratory drive to the ipsilateral phrenic nucleus (Goshgarian 2003). This pathway consists of axonal collaterals from uninjured, contralateral bulbospinal fibers that form monosynaptic projections onto phrenic motoneurons ipsilateral to the C2HS (Goshgarian et al. 1991; Moreno et al. 1992). This basic circuitry, however, does not take into account other neuronal constituents – interneurons in particular – that may affect the nature and extent of spontaneous recovery (i.e., the sCPP) in the chronically injured spinal cord.

2. Spinal interneurons and neuroplasticity following SCI

Increasing recognition of the neuroplastic reserve of the injured spinal cord has evolved in recent years (Fouad et al. 2008; Harkema 2008; Stein 2008; Blesch et al. 2009; Darian-Smith 2009), and spinal interneurons may play prominent roles in neural circuit remodelling and modulation of motoneuron excitability. For example, one emerging concept is that interneurons with axonal projections traversing multiple spinal levels (i.e., long propriospinal interneurons) can provide alternative substrates capable of mediating locomotion following incomplete lesions of the spinal cord (Bareyre et al. 2004; Courtine et al. 2008). Short (inter- or intrasegmental) propriospinal interneurons, on the other hand, may play critical roles in regulating the expression of spontaneous or therapeutically-driven plasticity by influencing motoneuron activity or adopting novel functional tasks (Jankowska 2001). As once stated, the injured spinal cord represents a “new anatomy” (Dimitrijevic et al. 1997), and interneurons may be an integral part of the altered circuitry. Because spinal interneurons also appear to impact upon plasticity in other functional domains (Krassioukov et al. 2002; de Groat et al. 2006; Llewellyn-Smith et al. 2006; Rabchevsky 2006; Zinck et al. 2006; Hou et al. 2008a; Hou et al. 2008b), the question arises whether such cells may be involved in spinal circuits affecting the expression of protracted, spontaneous respiratory plasticity.

3. Spinal interneurons: projections onto respiratory motoneurons

The diaphragm and the circuitry mediating its function (i.e., phrenic motor system) are key components of inspiration (Feldman 1986). Direct monosynaptic innervation of phrenic motoneurons by respiratory bulbospinal axons has been extensively documented in the rat (Ellenberger et al. 1988; Ellenberger et al. 1990). In cats and ferrets, however, both monosynaptic and polysynaptic pathways have been observed (Yates et al. 1999; Lois et al. 2009). Pre-phrenic interneurons (neurons with projections to phrenic motoneurons) have also been identified which appear to coordinate activity within phrenic and other circuits associated with accessory respiratory motoneuron pools (e.g. Aminoff et al. 1971; Sears 1990; Kirkwood et al. 1993). One suggested role for pre-phrenic interneurons is the inhibition of phrenic activity during expiration as shown in the cat (Douse et al. 1993). However, cervical interneurons seem to subserve heterogeneous functions (reviewed in Hilaire et al. 1997). In the cat, for example, interneurons in the lower, but not upper, cervical spinal cord appear to modulate respiratory

function during ventilatory-related behaviors such as cough (Grelot et al. 1993; Miller et al. 1993).

Although electrophysiological evidence has suggested some spinal interneurons are associated with respiration in the adult rat (see below and Section 9), very little attention has been given to their neuroanatomical representation within the phrenic motor system. Even less is known about the function of these cells which may differ from that shown in other species that possess respiratory behaviors (e.g., cough, emesis) not present in rats. Furthermore, previous anatomical studies of the CPP in the rat have dismissed an interneuronal contribution to recovery of phrenic activity in that model (Goshgarian et al. 1991; Moreno et al. 1992). However, Lipski et al. (1993) reported evidence for inspiratory neurons in the upper cervical spinal cord of adult rat. While the majority of those cells appeared to have long descending projections extending towards intercostal motoneurons, some axon collaterals were seen directed toward phrenic motoneurons. These findings are consistent with studies in the cat that provided electrophysiological demonstration of “upper cervical interneurons” (e.g. Lipski et al. 1986; Duffin et al. 1987; Nakazono et al. 1994). Collectively, those earlier findings suggest the presence of at least one population of cells in the adult rat spinal cord that may have the capability to influence respiratory, and in particular phrenic, recovery after SCI (see Section 8).

Beyond the study by Lipski et al. (1993), the only other anatomical indication of interneuronal projections onto phrenic motoneurons in rat was shown by Dobbins and Feldman. (1994), who employed a pseudorabies virus (PRV) retrograde transneuronal tracing approach. Their investigation, which primarily focused on identification of medullary neurons projecting onto the phrenic motoneuron pool, revealed interneurons in Rexed laminae VII and X at the second cervical segment and laminae VII and IX at the level of the phrenic motor pool. Given the time-course and pattern of second- and third-order PRV labeling, however, these authors could not definitively determine whether those spinal cells were second-order to phrenic motoneurons (pre-phrenic) or ones with ascending projections to the medulla (i.e., third-order neurons or greater). As the present review shows, both populations exist in the adult rat and refining the use of neuroanatomical methods available enables distinction between them.

4. Neuroanatomical evidence for spinal respiratory interneurons in adult rat

A re-evaluation of anatomical relationships between spinal interneurons and the phrenic nucleus of the rat was recently carried out in an effort to gain a greater understanding of the circuitry that may influence spontaneous recovery of phrenic motoneuron and ipsilateral diaphragm functions after C2HS. Our primary focus was on cervical interneurons projecting onto phrenic motoneurons. The aim of initial studies was to establish a baseline for the C2HS model by obtaining a more complete perspective of their numbers, distribution, and connectivity patterns in normal rats. These findings have been described in detail previously (Lane et al. 2008b) and are summarized below.

In the first set of experiments, PRV-Bartha was delivered to the left hemidiaphragm in normal adult female Sprague-Dawley rats. Phrenic motoneurons ipsilateral to the labeled diaphragm were infected with PRV 48 hours later, at which time no labeling was visible in any other spinal or supraspinal cell population. In addition, no PRV-positive cells were observed in the phrenic pool contralateral to the labeled hemidiaphragm. Approximately 64 hours following PRV delivery, and before labeling was visible in the brainstem, PRV-positive interneurons were observed throughout the cervical spinal cord below C2 ipsi- and contralateral to the labeled phrenic motoneurons (Fig. 1). Few PRV-positive interneurons were observed at C1-C2, suggesting limited connectivity within labeled phrenic motoneurons, as suggested by findings previously reported by Lipski et al. (1993). Combined transynaptic (using PRV) and

monosynaptic (using cholera toxin beta subunit) retrograde tracing distinguished interneurons from motoneurons (Lane et al. 2008b). Although phrenic motoneurons were labeled with both tracers, cells beyond the defined column of phrenic motoneurons were labeled with PRV only. This finding confirms that cells only labeled with PRV were transynaptically infected and thus operationally considered second-order to phrenic motoneurons (i.e., pre-phrenic or spinal premotor interneurons).

While the majority of second-order cells were observed in laminae VII and X (111 ± 24 ipsilateral to labeled phrenic pool; 48 ± 14 contralateral), an additional population was seen in the right and left dorsal horns (38 ± 15 ipsilateral; 6 ± 3 contralateral). These findings suggest that a more substantial population of pre-phrenic interneurons is present in the rat spinal cord than previously appreciated. In addition, we observed another population of PRV-infected cells that usually appeared at ~72 hours post-PRV delivery. These cells were positioned immediately lateral and medial to the labeled phrenic motoneuron pool. Since these interneurons were infected later, they were not included in our original quantitative analyses. However, as discussed below, they may represent a population of cells with potentially important functional implications (referred to in Section 9).

5. Spinal respiratory interneurons: connectivity with supraspinal centers

Electrophysiological studies in the cat have revealed medullary inspiratory drive to interneurons in the cervical (Bellingham et al. 1990) and thoracic spinal cord (Kirkwood et al. 1988). There has been some additional evidence in cat and ferret that respiratory interneurons in the cervical spinal cord receive parallel inputs from the vestibular nuclei (Anker et al. 2006) and also from medullary centers that are known to regulate diaphragm function (Yates et al. 1999; Lois et al. 2009). As noted below (Section 8), some electrophysiological evidence has been reported for polysynaptic pathways from the ventral respiratory column (VRC) to phrenic motoneurons in the rat. Correlative neuroanatomical confirmation of these polysynaptic pathways was recently provided by our laboratory (Lane et al. 2008b).

Anterograde tracing studies of projections from VRC neurons on one side of the medulla showed that in addition to direct ipsilateral projections to phrenic motoneurons, some fibers crossed the spinal midline (Goshgarian et al. 1991) and formed terminal arborizations within the contralateral phrenic pool. In addition, decussating fibers branched immediately lateral to the central canal (Goshgarian et al. 1991).

The possibility that VRC fibers innervate spinal interneurons associated with phrenic motoneurons was previously dismissed on the basis of negative transynaptic wheat germ agglutinin-horseradish peroxidase (WGA-HRP) tracing results (Moreno et al. 1992). These results have been replicated and could reflect different WGA-HRP vs. PRV tracing properties (see discussion in Lane et al. 2008b). This also is reflected by the fact that WGA-HRP appears to label only a subset of medullary neurons (e.g. Moreno et al. 1992) compared to results using PRV or rabies virus (Dobbins et al. 1994; Yates et al. 1999; Lois et al. 2009; Rice et al. 2009).

In subsequent experiments, the VRC was electrophysiologically identified by recording spontaneous, rhythmic activity in the medulla in phase with diaphragm electromyogram recordings (Lane et al. 2008b). Discrete iontophoretic injection of Miniruby (fluorescently labeled biotin dextran amine, 10,000kDa) into the region of the VRC and delivery of PRV to the ipsilateral hemi-diaphragm revealed a vast number of BDA-labeled projections to PRV-labeled phrenic motoneurons (Figure 2). To a lesser degree, VRC projections also were observed in the region of the contralateral phrenic pool (Figure 2). Of particular interest, these tracing studies provided evidence for VRC projections to the soma and dendrites of PRV

labeled, pre-phrenic interneurons around the central canal ipsi- and contralateral to the labeled phrenic pool (Lane et al. 2008b).

In addition to anterogradely labeled bulbospinal projections, delivery of Miniruby into the VRC resulted in some retrograde labeling. This revealed a separate population of neurons in the cervical spinal cord (Figure 2) with *ascending* projections to the region of the VRC (Lane et al. 2009). These cells were distributed bilaterally throughout the dorsal horn and medial spinal gray matter, predominantly in the upper cervical segments. Sixty-four hours following delivery of PRV to the left hemidiaphragm, no Miniruby-positive cells were infected with PRV. Therefore, it appears that the Miniruby-labeled neurons observed project to the medulla only and thus are not overlapping with the pre-phrenic interneurons described above. Previous work has supported the notion that some cervical interneurons may represent part of an *ascending* polysynaptic pathway mediating afferent input to supraspinal respiratory centers (Cleland et al. 1993). To our knowledge, this is the first anatomical indication of such spinal interneurons in the adult rat.

6. Changes in spinal respiratory interneurons following C2 hemisection: ipsilateral phrenic circuit

The next question addressed was whether any temporal remodeling of the phrenic circuitry occurs following C2HS as reflected by changes in the number or distribution of PRV labeled cells. PRV labeling via ipsilateral hemidiaphragm delivery was performed two weeks after injury based upon previous documentation of the initial emergence of the sCPP (Golder et al. 2005; Fuller et al. 2006; Fuller et al. 2008). As in spinal-intact rats, analysis of the number of PRV-positive interneurons relative to labeled phrenic motoneurons (ratio of secondary to primary cell labeling) was limited to 64 hours post-PRV delivery to the ipsilateral diaphragm. This method accounts for variation in labeling efficiency between animals (see Lane et al. 2008b for discussion).

Using this approach, the number of second-order neurons in contralateral laminae VII and X were significantly reduced, while the number and distributions of other pre-phrenic interneurons was unaltered (Lane et al. 2008b). More recent findings show that by 12 weeks following C2HS, the ratio of interneurons to motoneurons is significantly reduced in laminae VII and X both ipsi- and contralateral to the injury site (Lane et al. 2009). Meanwhile, the ratio of dorsal horn to phrenic motoneurons remained stable even 12 weeks following injury.

While a variety of studies have shown consistent evidence for spontaneous recovery of phrenic motoneuron excitability and breathing behavior after C2HS, the extent of such recovery remains modest for months post-injury (Fuller et al. 2008). Changes in laminae VII and X second-order cell labeling could indicate that reduced connectivity (Card et al. 1999) within the ipsilateral phrenic circuit may be involved, especially if some interneurons affected exert excitatory influences via a polysynaptic pathway.

Qualitative anterograde tracing observations in animals 2 weeks post-C2HS (Lane et al. 2009) have also revealed an apparent reduction in ipsi- and contralateral VRC projections to the ipsilateral phrenic motoneuron pool compared with what has been described in uninjured animals (compare Figures 2 and 4) (Lane et al. 2009). Qualitatively, VRC inputs to persisting pre-phrenic interneurons appeared to be predominantly from the contralateral side of the medulla, suggesting some of these interneurons may be part of the midline pathway typically considered to represent crossed monosynaptic projections (Goshgarian, 2003). These findings are consistent with previous retrograde tracing studies by Boulenguez et al. (2007) which showed that monosynaptic bulbospinal projections from both sides of the medulla to the region of the ipsilateral phrenic motor pool are dramatically reduced following C2HS. Together, these

observations suggest that there is insufficient innervation of the affected phrenic circuit by bulbospinal axons that arise predominantly from the contralateral VRC.

7. Plasticity, spinal interneurons, and compensation in other respiratory circuits

Although studies of respiratory plasticity following SCI have focused on recovery of ipsilateral phrenic function following C2HS (i.e., “restorative” neuroplasticity), the functional improvement is limited (as noted earlier), and respiratory deficits persist (Fuller et al. 2008). Phrenic nerve recordings (Fuller et al. 2008) and diaphragm EMG data (Lane, Fuller, Reier – unpublished) show that activity ipsilateral to C2HS remains minimal even 12 weeks following injury. In contrast, compensatory activity in other spinal respiratory circuits can contribute to altered post-injury breathing behavior. It is well-known that following C2HS, animals adopt a rapid, shallow ventilatory pattern in order to maintain appropriate blood-gas levels (Goshgarian et al. 1986; Golder et al. 2001b; Fuller et al. 2008; Lane et al. 2008a). Activity in the contralateral phrenic motoneuron pool is increased following C2HS (Golder et al. 2003; Fuller et al. 2006) and most likely contributes to this altered pattern of breathing. This contralateral activity also may reflect an attempt to partially compensate for ipsilateral respiratory deficit as seen under other experimental conditions (Katagiri et al. 1994; Miyata et al. 1995; Prakash et al. 1999; Sandhu et al. 2009).

At present the direct effect compensation may have on the extent and onset of ipsilateral “restorative plasticity” associated with the sCPP remains poorly defined. While contralateral phrenic output is increased soon after injury, there is some evidence for a reduction in this output as ipsilateral phrenic activity spontaneously recovers (Golder et al. 2001a; Fuller et al. 2006). The altered pattern of activity suggests a close communication between right and left phrenic circuits that we recently demonstrated neuroanatomically in uninjured rats.

Using two variants of the PRV Bartha virus (PRV152 and 614, resulting in neuronal expression of green fluorescent protein and red fluorescent protein respectively) (Lane et al. 2008b), we found bilateral patterns of pre-phrenic interneuron labeling as previously observed with PRV 152 only but which now included a subset of neurons labeled with both PRV variants. This indicated the presence of a population of spinal interneurons that could integrate phrenic circuits on opposite sides of the spinal cord. These cells may partially provide an unrecognized anatomical basis for patterns of synchronized bursting activity between right and left phrenic circuits after C2HS (Sandhu et al. 2009).

We next hypothesized that some spinal premotor interneurons also may integrate with other neuronal networks, given the range of reflexes observed between phrenic, other respiratory (e.g. intercostal (Decima et al. 1967; Decima et al. 1969; Knill et al. 1976; Shannon 1980; Bellingham 1999), abdominal (Shannon 1980)), and non-respiratory circuits (e.g. postural, locomotor (Viala et al. 1979; Eldridge et al. 1981; Viala 1986)). Again, using dual-tracing studies with PRV152 and PRV614, we retrogradely labeled phrenic and intercostal motoneuron pools (Lane et al. 2008b). Tracing of the circuit innervating intercostal muscles revealed a population of second-order spinal cells with a distribution pattern similar to that of pre-phrenic interneurons. These studies also confirmed our hypothesis by revealing a population of interneurons that integrate ipsilateral phrenic and intercostal circuits by virtue of dual PRV labeling.

8. Changes in spinal respiratory interneurons following C2 hemisection: contralateral phrenic circuit

Given functional changes occurring contralateral to the injury, the question arises whether remodeling of the contralateral phrenic circuit is associated with compensatory phrenic motoneuron responses to C2HS. To address this issue, we recently initiated studies in which PRV was applied to the contralateral hemidiaphragm to transynaptically label the associated phrenic circuit following C2HS (Lane et al. 2009). This resulted in robust second-order infection of interneurons, similar in laminar distribution to that seen in uninjured animals. However, while the number of pre-phrenic interneurons at the C1-2 level is minimal in spinal-intact rats, interneurons associated with the contralateral phrenic motoneuron pool following C2HS were consistently observed at and above the C2 level of injury on both sides of the cord. These initial findings suggest possible recruitment of cells that normally are poorly, if at all, connected with the phrenic circuitry. If these cells represent the same neurons as identified by Lipski et al (1993) in the uninjured spinal cord, future anterograde tracing studies should demonstrate these cells also receive input from the medullary respiratory neurons as seen in rat (Lipski et al. 1994) and cat (Hoskin et al. 1987a; Hoskin et al. 1987b; Mateika et al. 1989). Accordingly, these interneurons would be anatomically positioned to not only mediate increased compensatory drive to the contralateral phrenic motoneuron pool, but also to intercostal or abdominal circuits after C2HS.

9. Respiratory interneurons in the rat: neuroanatomical and electrophysiological correlates

A few studies have reported cervical spinal interneurons in the adult rat which show respiratory-related discharge (Lipski et al. 1993; Hayashi et al. 2003), and our recent correlative neuroanatomical data (see above) provide a neural substrate basis for those findings. These and other observations in our laboratory and others, also offer an opportunity to speculate on potential roles of interneurons in post-C2HS and other forms of spinal respiratory plasticity.

VRC projections and spinal respiratory interneurons

Of particular interest is our evidence for putative VRC projections to pre-phrenic interneurons seen *below* C2. Those data are consistent with previously reported electrophysiological results (Ling et al. 1995; Tian et al. 1996). For example, Ling et al. (1995) showed electrical stimulation of the ventral funiculus on one side of the spinal cord could evoke compound action potentials in the contralateral phrenic nerve with both short (i.e. ~ 1.0 ms) and relatively long onset latencies (i.e. 5-7 ms). The latter were serotonin-dependent and suggested the presence of polysynaptic inputs to phrenic motoneurons (Ling et al. 1995).

Additional evidence was subsequently reported by Hayashi et al. (2003) who studied the contribution of spinal mechanisms to short-term potentiation (STP) of phrenic motoneuron activity. High-frequency stimulation of the lateral funiculus immediately below a complete transection of the C₁ spinal cord elicited phrenic STP that peaked several seconds post-stimulation. One explanation for the response latency noted was that interneurons may be interposed between bulbospinal projections and phrenic motoneurons. This was supported by demonstration of extracellular recordings at the level of the phrenic nucleus which showed tonic and superimposed, phasic inspiratory activity that could be amplified by orthodromic spinal stimulation. No effect was obtained with supramaximal antidromic activation of the ipsilateral phrenic nerve thus suggesting these cells were not phrenic motoneurons (Hayashi et al. 2003).

Recent electrophysiological observations in our laboratory corroborate and extend the findings reported by Hayashi et al. (2003). As shown in Figure 5, we have recently obtained virtually identical extracellular recordings (compare Figure 10 in Hayashi et al., 2003) of cells at the level of the phrenic nucleus in non-spinalized rats which were not activated with antidromic stimulation of the ipsilateral phrenic nerve. Moreover, the tonic discharge pattern of this neuron during normoxic “baseline” conditions (Fig. 5i) is not observed in phrenic motoneurons under comparable recording conditions (Lee et al. 2009) providing further evidence that the recording is from a cervical interneuron. Interestingly, the activity of this neuron was enhanced during hypoxia (Figure 5). Therefore, some pre-phrenic interneurons may be stimulated under conditions of heightened inspiratory drive. This supports the suggestion made by Hayashi et al. (2003) that some spinal interneurons may be interposed between medullary centers and phrenic motoneurons serving as an amplifier to “increase the gain of synaptic input to motoneurons”. From a neuroanatomical perspective, however, it should be noted that our evidence for an interneuronal VRC-to-phrenic motoneuron relay is thus far limited to cells in Rexed laminae VII and X, whereas the electrophysiological data presented above have been obtained at the level of the phrenic motoneuron pool itself. As noted earlier (see Section 4) and shown in Figure 1, indications of second-order PRV-infected cells have been observed in that region. For technical reasons (Lane et al. 2008b), we have thus far chosen to be conservative in interpreting them as pre-phrenic interneurons. In view of the neurophysiological evidence cited, further study of these cells is warranted.

It is conceivable that the initial physiological stress associated with unilateral denervation of a phrenic motoneuron pool may result not only in activation of a crossed monosynaptic VRC pathway, but a polysynaptic circuit as well. Given the observed response of some interneurons to hypoxia, however, it may be possible these cells play a greater role in responses to chemical challenge after SCI. In that regard, the Hayashi et al (2003) data represent potential interneuronal contributions to phrenic motoneuron plasticity in an acute, high cervical transection model. This type of interneuronal relay may thus be involved in the CPP/sCPP, as well as other expressions of phrenic motoneuron plasticity including, but not limited to, long-term facilitation in response to intermittent hypoxia (Mitchell et al. 2001; Mitchell et al. 2003). To date, proposed pharmacological mechanisms underlying the effects of acute and chronic intermittent hypoxia have focused primarily on phrenic motoneuron, although potential spinal interneuron contributions have been considered (Golder et al. 2008).

Spinal respiratory pattern generation and interneurons

A potentially significant contribution of interneurons to respiratory recovery in the C2HS model has recently been indicated by an intriguing study published by Alilain et al. (2008). Immediately after creating C2HS lesions, a Sindbis viral vector expressing Channelrhodopsin-2 was injected into the spinal cord in proximity to the ipsilateral phrenic motoneuron pool. Four days later, the transduced neurons, most of which were interneurons (Alilain and Silver, personal communication), were exposed to intermittent intervals of photostimulation which resulted in a long-lasting (i.e., beyond the stimulus) synchronous, bilateral diaphragm contraction in the absence of descending respiratory drive. Pharmacological studies revealed that the light-induced activation of phrenic activity in these experiments was NMDA receptor-dependent. Furthermore, some of the transduced neurons had axons that extended across the midline, as has been described for PRV labeled interneurons (Lane et al. 2008b).

In addition to the demonstration of pre-phrenic interneurons with bilateral projections to right and left phrenic circuits, the results of Alilain et al. (2008) raise speculation for the existence of a spinal oscillator that can impart respiratory rhythm. Although dominant respiratory rhythm generation resides at medullary levels (Feldman et al. 2006), some evidence for existence of a

spinal respiratory pattern generator also has been reported (e.g. Palissés et al. 1987). However, the basis for this phenomenon has been considered equivocal in terms of the underlying circuitry and mechanisms (reviewed in Feldman (1986)). It is nevertheless tempting to consider that the presence of a spinal pattern generator-like circuitry that is not easily detected in the intact spinal cord may be revealed after injury.

Spinal interneurons and recovery of ipsilateral phrenic motoneuron activity

Employing cross-correlation histogram analyses, Duffin and Alpen (1995) previously demonstrated in spinal-intact rats substantial bilateral synchronous excitation of right and left phrenic motoneuron pools via descending projections from VRC neurons. As discussed in detail elsewhere in this Special Issue (Sandhu et al. 2009), recent analyses of cross-correlation data from rats, 12-weeks post-C2HS, showed in some cases a delayed activation of ipsilateral versus contralateral phrenic motoneurons. While other interpretations must be considered (see discussion in Sandhu et al. 2009) the cross-correlation data suggest that the neural substrate associated with recovered motoneuron function may entail polysynaptic, as well as monosynaptic bulbospinal inputs.

Spinal interneurons and primary afferents

It has been previously demonstrated that acute (Goshgarian 1981), as well as chronic (Fuller et al. 2002), dorsal rhizotomies contralateral to C2HS lesions can accelerate functional recovery in the ipsilateral phrenic circuit. This *induced* restoration was attributed to removal of phrenic afferent projections that innervate phrenic motoneurons on the opposite side of the cord (Goshgarian 1981). More recent pharmacological studies have shown that the disinhibition observed with contralateral dorsal rhizotomy is GABA dependent (Zimmer et al. 2007). This GABAergic inhibition of phrenic activity on the opposite side of the spinal cord was postulated to occur via dorsal horn neurons (Zimmer et al. 2007). Results from neuroanatomical tracing have identified candidate interneurons in the dorsal horn (with either ascending projection toward the VRC (Section 5) or direct projections onto phrenic motoneurons) that support those findings. The neurotransmitter phenotype(s) of those cells and their relationship to primary afferent input, however, remains to be determined. In that regard, further mapping of the phrenic motor circuit will provide greater definition for pharmacological/molecular manipulation of the sCPP.

10. Concluding Remarks

Increasing attention to neuroplasticity in the injured spinal cord under experimental and clinical conditions has underscored a need for greater understanding of associated neural substrates. Such information can facilitate better definition of therapeutic targets and underlying mechanisms not only in relation to spontaneous recovery processes, but also therapeutically-driven repair. Thus far, the neural circuits mediating most forms of neuroplasticity in the injured spinal cord have proven challenging to identify. In contrast, previous anatomical studies of the CPP have elegantly described a comparatively simple anatomical substrate as a model for respiratory recovery in a partial spinal injury model. Crossed, monosynaptic VRC projections appear to play a key role in mediating recovery of ipsilateral phrenic motoneuron activity. However, the collective neuroanatomical findings described in this review now draw attention to the possibility that the underlying neural substratum associated with *spontaneous* recovery in the phrenic circuit may be more complex than previously envisioned.

As our transneuronal PRV findings have shown, a substantial contingent of bilaterally distributed interneurons is associated with phrenic motoneurons on each side of the spinal cord. More detailed anatomical-neurophysiological studies are required, but our anatomical findings to date are consistent with past and more recent evidence that suggest some spinal interneurons

could be involved in mediating respiratory function in normal and spinal-lesioned rats. Furthermore, the apparent reduction in PRV labeling of some pre-phrenic cells after C2HS suggests a possible impact on the extent of functional recovery in the ipsilateral phrenic circuit.

Formulating hypotheses related to such potentially important neuronal dynamics would be difficult without the benefit of a neuroanatomical framework. One hypothesis suggested by data obtained thus far is that recovery of ipsilateral phrenic motoneuron activity after C2HS involves multiple, and not necessarily mutually exclusive, neural substrates. The induced CPP represents a situation in which the latent pathway can be activated in response to asphyxia following C2HS and contralateral phrenicotomy (Goshgarian, 2003). Under such circumstances, monosynaptic projections would represent the most effective route for enhanced excitatory drive to the ipsilateral phrenic motoneuron pool. On the other hand, in the case of spontaneous recovery, an early indication of restoration is low amplitude rhythmic phrenic motoneuron bursting activity. While this still could be mediated by a crossed, monosynaptic projection system, cross-correlation and Channelrhodopsin data reviewed above imply gradual recruitment of other neuronal populations and unmasking of an endogenous spinal respiratory pattern generator, respectively.

The degree to which a particular animal model permits extrapolations to the human may be critically dependent upon neuroanatomical similarities or differences (Blesch et al. 2008; Darian-Smith 2009). While aspects of respiratory function and neuroanatomy in the rodent are relatively unique (e.g. Duffin et al. 1995), the anatomical organization of spinal circuits in rat now appears more comparable to that described in other species (Yates et al. 1999; Billig et al. 2000; Lois et al. 2009). Our recent studies of the mouse phrenic circuitry (Qiu et al. 2009) demonstrate very similar features which are important given recent reports of apparent CPP-like plasticity in that animal model (Minor et al. 2006).

To conclude, we have provided clear anatomical evidence for spinal interneurons in the adult rat with projections to phrenic and intercostal motoneurons and ascending projections in proximity to the VRC. Such observations assume special significance since electrophysiological studies in a number of species have shown that spinal interneurons with segmental or spino-medullary projections can modulate normal respiratory activity. From a clinical perspective, discussion of respiratory control in the human and the role of polysynaptic drive to inspiratory motoneurons (Butler 2007; Butler et al. 2008) outline the need for a better understanding of the connectivity of spinal interneurons with these circuits. Since the rat is often the model of choice for studies of respiratory neuroplasticity, the cross-species similarities of spinal circuitry strengthens the value of data in the rat that could be subsequently translated to higher animal models of spinal cord plasticity.

Acknowledgments

Support for this work was provided by grants from the National Institutes of Health (NIH): NIH NS054025 (PJR) and NIH HD052682, NIH 1R01HD052682-01A1 (DDF).. Additional support was also provided by the Anne and Oscar Lackner Chair in Medicine (PJR) and the Craig H. Neilsen Foundation (MAL), and the University of Florida (KZL). PRV152 was provided to us by L. W. Enquist, Princeton University as a service of the National Center for Experimental Neuroanatomy with Neurotropic Viruses (NCRR P40 RR0118604). PRV614 was generously donated by Dr Bruce Banfield at the University of Colorado. Special thanks go to Barbara O'Steen, Alex Jones and Forest Hunsaker for their excellent technical support.

References

- Alilain WJ, Li X, Horn KP, Dhingra R, Dick TE, Herlitze S, Silver J. Light-induced rescue of breathing after spinal cord injury. *J Neurosci* 2008;28:11862–11870. [PubMed: 19005051]
- Aminoff MJ, Sears TA. Spinal integration of segmental, cortical and breathing inputs to thoracic respiratory motoneurons. *J Physiol* 1971;215:557–575. [PubMed: 4336048]

- Anker AR, Sadacca BF, Yates BJ. Vestibular inputs to propriospinal interneurons in the feline C1-C2 spinal cord projecting to the C5-C6 ventral horn. *Exp Brain Res* 2006;170:39–51. [PubMed: 16328293]
- Bareyre FM, Kerschensteiner M, Raineteau O, Mettenleiter TC, Weinmann O, Schwab ME. The injured spinal cord spontaneously forms a new intraspinal circuit in adult rats. *Nat Neurosci* 2004;7:269–277. [PubMed: 14966523]
- Bellingham MC. Synaptic inhibition of cat phrenic motoneurons by internal intercostal nerve stimulation. *J Neurophysiol* 1999;82:1224–1232. [PubMed: 10482742]
- Bellingham MC, Lipski J. Respiratory interneurons in the C5 segment of the spinal cord of the cat. *Brain Research* 1990;533:141–146. [PubMed: 2085725]
- Billig I, Foris JM, Enquist LW, Card JP, Yates BJ. Definition of neuronal circuitry controlling the activity of phrenic and abdominal motoneurons in the ferret using recombinant strains of pseudorabies virus. *J Neurosci* 2000;20:7446–7454. [PubMed: 11007904]
- Blesch A, Tuszynski MH. Spinal cord injury: plasticity, regeneration and the challenge of translational drug development. *Trends Neurosci* 2008;32:41–47. [PubMed: 18977039]
- Blesch A, Tuszynski MH. Spinal cord injury: plasticity, regeneration and the challenge of translational drug development. *Trends Neurosci* 2009;32:41–47. [PubMed: 18977039]
- Boulenguez P, Gauthier P, Kastner A. Respiratory neuron subpopulations and pathways potentially involved in the reactivation of phrenic motoneurons after C2 hemisection. *Brain Res* 2007;1148:96–104. [PubMed: 17379194]
- Butler JE. Drive to the human respiratory muscles. *Respir Physiol Neurobiol* 2007;159:115–126. [PubMed: 17660051]
- Butler JE, Gandevia SC. The output from human inspiratory motoneurone pools. *J Physiol* 2008;586:1257–1264. [PubMed: 17974589]
- Card JP, Enquist LW, Moore RY. Neuroinvasiveness of pseudorabies virus injected intracerebrally is dependent on viral concentration and terminal field density. *J Comp Neurol* 1999;407:438–452. [PubMed: 10320223]
- Cleland CL, Getting PA. Respiratory-modulated and phrenic afferent-driven neurons in the cervical spinal cord (C4-C6) of the fluorocarbon-perfused guinea pig. *Exp Brain Res* 1993;93:307–311. [PubMed: 8491269]
- Courtine G, Song B, Roy RR, Zhong H, Herrmann JE, Ao Y, Qi J, Edgerton VR, Sofroniew MV. Recovery of supraspinal control of stepping via indirect propriospinal relay connections after spinal cord injury. *Nat Med* 2008;14:69–74. [PubMed: 18157143]
- Darian-Smith C. Synaptic plasticity, neurogenesis, and functional recovery after spinal cord injury. *Neuroscientist* 2009;15:149–165. [PubMed: 19307422]
- de Groat WC, Yoshimura N. Mechanisms underlying the recovery of lower urinary tract function following spinal cord injury. *Prog Brain Res* 2006;152:59–84. [PubMed: 16198694]
- Decima EE, von Euler C, Thoden U. Spinal intercostal-phrenic reflexes. *Nature* 1967;214:312–313. [PubMed: 5231342]
- Decima EE, von Euler C, Thoden U. Intercostal-to-phrenic reflexes in the spinal cat. *Acta Physiol Scand* 1969;75:568–579. [PubMed: 4243231]
- Dimitrijevic MR, McKay WB, Sherwood AM. Motor control physiology below spinal cord injury: residual volitional control of motor units in paretic and paralyzed muscles. *Adv Neurol* 1997;72:335–345. [PubMed: 8993710]
- Dobbins EG, Feldman JL. Brainstem network controlling descending drive to phrenic motoneurons in rat. *J Comp Neurol* 1994;347:64–86. [PubMed: 7798382]
- Douse MA, Duffin J. Axonal projections and synaptic connections of C5 segment expiratory interneurons in the cat. *J Physiol* 1993;470:431–444. [PubMed: 8308735]
- Duffin J, Hoskin RW. Intracellular recordings from upper cervical inspiratory neurons in the cat. *Brain Res* 1987;435:351–354. [PubMed: 3427463]
- Duffin J, van Alphen J. Bilateral connections from ventral group inspiratory neurons to phrenic motoneurons in the rat determined by cross-correlation. *Brain Res* 1995;694:55–60. [PubMed: 8974664]

- Eldridge FL, Gill-Kumar P, Millhorn DE, Waldrop TG. Spinal inhibition of phrenic motoneurons by stimulation of afferents from peripheral muscles. *J Physiol* 1981;311:67–79. [PubMed: 7264986]
- Ellenberger HH, Feldman JL. Monosynaptic transmission of respiratory drive to phrenic motoneurons from brainstem bulbospinal neurons in rats. *J Comp Neurol* 1988;269:47–57. [PubMed: 3361003]
- Ellenberger HH, Feldman JL, Goshgarian HG. Ventral respiratory group projections to phrenic motoneurons: electron microscopic evidence for monosynaptic connections. *J Comp Neurol* 1990;302:707–714. [PubMed: 1707065]
- Feldman, JL. *Handbook of Physiology - The Nervous System IV*. 1986. Neurophysiology of breathing in mammals.; p. 463-524.
- Feldman JL, Del Negro CA. Looking for inspiration: new perspectives on respiratory rhythm. *Nat Rev Neurosci* 2006;7:232–242. [PubMed: 16495944]
- Fouad K, Tse A. Adaptive changes in the injured spinal cord and their role in promoting functional recovery. *Neurol Res* 2008;30:17–27. [PubMed: 18387259]
- Fuller DD, Doperalski NJ, Dougherty BJ, Sandhu MS, Bolser DC, Reier PJ. Modest spontaneous recovery of ventilation following chronic high cervical hemisection in rats. *Exp Neurol* 2008;211:97–106. [PubMed: 18308305]
- Fuller DD, Golder FJ, Olson EB Jr. Mitchell GS. Recovery of phrenic activity and ventilation after cervical spinal hemisection in rats. *J Appl Physiol* 2006;100:800–806. [PubMed: 16269524]
- Fuller DD, Johnson SM, Johnson RA, Mitchell GS. Chronic cervical spinal sensory denervation reveals ineffective spinal pathways to phrenic motoneurons in the rat. *Neurosci Lett* 2002;323:25–28. [PubMed: 11911982]
- Fuller DD, Johnson SM, Olson EB Jr. Mitchell GS. Synaptic pathways to phrenic motoneurons are enhanced by chronic intermittent hypoxia after cervical spinal cord injury. *J Neurosci* 2003;23:2993–3000. [PubMed: 12684486]
- Golder FJ, Fuller DD, Davenport PW, Johnson RD, Reier PJ, Bolser DC. Respiratory motor recovery after unilateral spinal cord injury: eliminating crossed phrenic activity decreases tidal volume and increases contralateral respiratory motor output. *J Neurosci* 2003;23:2494–2501. [PubMed: 12657710]
- Golder FJ, Mitchell GS. Spinal synaptic enhancement with acute intermittent hypoxia improves respiratory function after chronic cervical spinal cord injury. *J Neurosci* 2005;25:2925–2932. [PubMed: 15772352]
- Golder FJ, Ranganathan L, Satriotomo I, Hoffman M, Lovett-Barr MR, Watters JJ, Baker-Herman TL, Mitchell GS. Spinal adenosine A2a receptor activation elicits long-lasting phrenic motor facilitation. *J Neurosci* 2008;28:2033–2042. [PubMed: 18305238]
- Golder FJ, Reier PJ, Bolser DC. Altered respiratory motor drive after spinal cord injury: supraspinal and bilateral effects of a unilateral lesion. *J Neurosci* 2001a;21:8680–8689. [PubMed: 11606656]
- Golder FJ, Reier PJ, Davenport PW, Bolser DC. Cervical spinal cord injury alters the pattern of breathing in anesthetized rats. *J Appl Physiol* 2001b;91:2451–2458. [PubMed: 11717204]
- Goshgarian HG. The role of cervical afferent nerve fiber inhibition of the crossed phrenic phenomenon. *Exp Neurol* 1981;72:211–225. [PubMed: 7202624]
- Goshgarian HG. The crossed phrenic phenomenon: a model for plasticity in the respiratory pathways following spinal cord injury. *J Appl Physiol* 2003;94:795–810. [PubMed: 12531916]
- Goshgarian HG, Ellenberger HH, Feldman JL. Decussation of bulbospinal respiratory axons at the level of the phrenic nuclei in adult rats: a possible substrate for the crossed phrenic phenomenon. *Exp Neurol* 1991;111:135–139. [PubMed: 1984430]
- Goshgarian HG, Moran MF, Prcevski P. Effect of cervical spinal cord hemisection and hemidiaphragm paralysis on arterial blood gases, pH, and respiratory rate in the adult rat. *Exp Neurol* 1986;93:440–445. [PubMed: 3089833]
- Grelot L, Milano S, Portillo F, Miller AD. Respiratory interneurons of the lower cervical (C4–C5) cord: membrane potential changes during fictive coughing, vomiting, and swallowing in the decerebrate cat. *Pflugers Arch* 1993;425:313–320. [PubMed: 8309792]
- Harkema SJ. Plasticity of interneuronal networks of the functionally isolated human spinal cord. *Brain Res Rev* 2008;57:255–264. [PubMed: 18042493]

- Hayashi F, Hinrichsen CF, McCrimmon DR. Short-term plasticity of descending synaptic input to phrenic motoneurons in rats. *J Appl Physiol* 2003;94:1421–1430. [PubMed: 12482770]
- Hilaire, G.; Monteau, R. Brainstem and spinal control of respiratory muscles during breathing.. In: Miller, AD.; Bianchi, AL.; Bishop, BP., editors. *Neural control of the respiratory muscles*. CRC; New York: 1997. p. 91-105.
- Hoskin R, Duffin J. Excitation of upper cervical inspiratory neurons by inspiratory neurons of the nucleus tractus solitarius in the cat. *Exp Neurol* 1987a;95:126–141. [PubMed: 3792473]
- Hoskin RW, Duffin J. Excitation of upper cervical inspiratory neurons by inspiratory neurons of the nucleus retroambigualis in the cat. *Exp Neurol* 1987b;98:404–417. [PubMed: 3666086]
- Hou S, Duale H, Cameron AA, Abshire SM, Lyttle TS, Rabchevsky AG. Plasticity of lumbosacral propriospinal neurons is associated with the development of autonomic dysreflexia after thoracic spinal cord transection. *J Comp Neurol* 2008a;509:382–399. [PubMed: 18512692]
- Hou S, Duale H, Rabchevsky AG. Intraspinal sprouting of unmyelinated pelvic afferents after complete spinal cord injury is correlated with autonomic dysreflexia induced by visceral pain. *Neuroscience* 2008b;159:369–379. [PubMed: 19146928]
- Jankowska E. Spinal interneuronal systems: identification, multifunctional character and reconfigurations in mammals. *J Physiol* 2001;533:31–40. [PubMed: 11351010]
- Katagiri M, Young RN, Platt RS, Kieser TM, Easton PA. Respiratory muscle compensation for unilateral or bilateral hemidiaphragm paralysis in awake canines. *J Appl Physiol* 1994;77:1972–1982. [PubMed: 7836225]
- Kirkwood PA, Munson JB, Sears TA, Westgaard RH. Respiratory interneurons in the thoracic spinal cord of the cat. *J Physiol* 1988;395:161–192. [PubMed: 3411478]
- Kirkwood PA, Schmid K, Sears TA. Functional identities of thoracic respiratory interneurons in the cat. *J Physiol* 1993;461:667–687. [PubMed: 8350279]
- Knill R, Bryan AC. An intercostal-phrenic inhibitory reflex in human newborn infants. *J Appl Physiol* 1976;40:352–356. [PubMed: 931849]
- Krassioukov AV, Johns DG, Schramm LP. Sensitivity of sympathetically correlated spinal interneurons, renal sympathetic nerve activity, and arterial pressure to somatic and visceral stimuli after chronic spinal injury. *J Neurotrauma* 2002;19:1521–1529. [PubMed: 12542854]
- Lane MA, Fuller DD, White TE, Reier PJ. Respiratory neuroplasticity and cervical spinal cord injury: translational perspectives. *Trends Neurosci* 2008a;31:538–547. [PubMed: 18775573]
- Lane MA, Jones AL, O'Steen BE, Hunsaker FL, Vavrousek J, Salazar K, Fuller DD, Reier PJ. Pre-phrenic interneurons as an anatomical substrate for plasticity following cervical spinal cord injury (SCI) in the adult rat. *FASEB J* 2009;23:834.5.
- Lane MA, White TE, Coutts MA, Jones AL, Sandhu MS, Bloom DC, Bolser DC, Yates BJ, Fuller DD, Reier PJ. Cervical prephrenic interneurons in the normal and lesioned spinal cord of the adult rat. *J Comp Neurol* 2008b;511:692–709. [PubMed: 18924146]
- Lee KZ, Reier PJ, Fuller DD. Phrenic motoneuron discharge patterns during hypoxia-induced short term potentiation in rats. *J Neurophysiol*. 2009
- Ling L, Bach KB, Mitchell GS. Phrenic responses to contralateral spinal stimulation in rats: effects of old age or chronic spinal hemisection. *Neurosci Lett* 1995;188:25–28. [PubMed: 7783972]
- Lipski J, Duffin J. An electrophysiological investigation of propriospinal inspiratory neurons in the upper cervical cord of the cat. *Exp Brain Res* 1986;61:625–637. [PubMed: 3956619]
- Lipski J, Duffin J, Kruszewska B, Zhang X. Upper cervical inspiratory neurons in the rat: an electrophysiological and morphological study. *Exp Brain Res* 1993;95:477–487. [PubMed: 8224074]
- Lipski J, Zhang X, Kruszewska B, Kanjhan R. Morphological study of long axonal projections of ventral medullary inspiratory neurons in the rat. *Brain Res* 1994;640:171–184. [PubMed: 8004446]
- Llewellyn-Smith IJ, Weaver LC, Keast JR. Effects of spinal cord injury on synaptic inputs to sympathetic preganglionic neurons. *Prog Brain Res* 2006;152:11–26. [PubMed: 16198690]
- Lois JH, Rice CD, Yates BJ. Neural circuits controlling diaphragm function in the cat revealed by transneuronal tracing. *J Appl Physiol* 2009;106:138–152. [PubMed: 18974365]
- Mateika JH, Duffin J. The connections from botzinger expiratory neurons to upper cervical inspiratory neurons in the cat. *Exp Neurol* 1989;104:138–146. [PubMed: 2707362]

- Miller AD, Yates BJ. Evaluation of role of upper cervical inspiratory neurons in respiration, emesis and cough. *Brain Research* 1993;606:143–147. [PubMed: 8461996]
- Minor KH, Akison LK, Goshgarian HG, Seeds NW. Spinal cord injury-induced plasticity in the mouse-The crossed phrenic phenomenon. *Exp Neurol* 2006;200:486–495. [PubMed: 16631169]
- Mitchell GS, Baker TL, Nanda SA, Fuller DD, Zabka AG, Hodgeman BA, Bavis RW, Mack KJ, Olson EB Jr. Invited review: Intermittent hypoxia and respiratory plasticity. *J Appl Physiol* 2001;90:2466–2475. [PubMed: 11356815]
- Mitchell GS, Johnson SM. Neuroplasticity in respiratory motor control. *J Appl Physiol* 2003;94:358–374. [PubMed: 12486024]
- Miyata H, Zhan WZ, Prakash YS, Sieck GC. Myoneural interactions affect diaphragm muscle adaptations to inactivity. *J Appl Physiol* 1995;79:1640–1649. [PubMed: 8594024]
- Moreno DE, Yu XJ, Goshgarian HG. Identification of the axon pathways which mediate functional recovery of a paralyzed hemidiaphragm following spinal cord hemisection in the adult rat. *Exp Neurol* 1992;116:219–228. [PubMed: 1375167]
- Nakazono Y, Aoki M. Excitatory connections between upper cervical inspiratory neurons and phrenic motoneurons in cats. *J Appl Physiol* 1994;77:679–683. [PubMed: 8002514]
- Nantwi KD, El-Bohy AA, Schrimsher GW, Reier PJ, Goshgarian H. Spontaneous functional recovery in a paralyzed hemidiaphragm following upper cervical spinal cord injury in adult rats. *Neurorehab Neural Repair* 1999;13:225–234.
- Palisses R, Viala D. [Existence of respiratory interneurons in the cervical spinal cord of the rabbit]. *C R Acad Sci III* 1987;305:321–324. [PubMed: 3115504]
- Prakash YS, Miyata H, Zhan WZ, Sieck GC. Inactivity-induced remodeling of neuromuscular junctions in rat diaphragmatic muscle. *Muscle & Nerve* 1999;22:30–319.
- Qiu K, Lane MA, Fuller DD, Reier PJ. Neuroanatomical characterization of the phrenic nucleus in adult mice. *FASEB J* 2009;23:783.2. [PubMed: 18948382]
- Rabchevsky AG. Segmental organization of spinal reflexes mediating autonomic dysreflexia after spinal cord injury. *Prog Brain Res* 2006;152:265–274. [PubMed: 16198706]
- Rice CD, Lois JH, Kerman IA, Yates BJ. Localization of serotonergic neurons that participate in regulating diaphragm activity in the cat. *Brain Res.* 2009
- Sandhu MS, Dougherty BJ, Lane MA, Bolser DC, Kirkwood PA, Reier PJ, Fuller DD. Respiratory recovery following high cervical hemisection. *Respir Physiol Neurobiol.* 2009In Press
- Sears TA. Central rhythm generation and spinal integration. *Chest* 1990;97:45S–51S. [PubMed: 2407462]
- Shannon R. Intercostal and abdominal muscle afferent influence on medullary dorsal respiratory group neurons. *Respir Physiol* 1980;39:73–94. [PubMed: 6244609]
- Stein RB. The plasticity of the adult spinal cord continues to surprise. *J Physiol* 2008;586:2823. [PubMed: 18556723]
- Tian GF, Duffin J. Spinal connections of ventral-group bulbospinal inspiratory neurons studied with cross-correlation in the decerebrate rat. *Exp Brain Res* 1996;111:178–186. [PubMed: 8891649]
- Viala D. Evidence for direct reciprocal interactions between the central rhythm generators for spinal “respiratory” and locomotor activities in the rabbit. *Exp Brain Res* 1986;63:225–232. [PubMed: 3758245]
- Viala D, Vidal C, Freton E. Coordinated rhythmic bursting in respiratory and locomotor muscle nerves in the spinal rabbit. *Neurosci Lett* 1979;11:155–159. [PubMed: 460684]
- Vinit S, Stamegna JC, Boulenguez P, Gauthier P, Kastner A. Restorative respiratory pathways after partial cervical spinal cord injury: role of ipsilateral phrenic afferents. *Eur J Neurosci* 2007;25:3551–3560. [PubMed: 17610574]
- Yates BJ, Smail JA, Stocker SD, Card JP. Transneuronal tracing of neural pathways controlling activity of diaphragm motoneurons in the ferret. *Neuroscience* 1999;90:1501–1513. [PubMed: 10338316]
- Zimmer MB, Goshgarian HG. GABA, not glycine, mediates inhibition of latent respiratory motor pathways after spinal cord injury. *Exp Neurol* 2007;203:493–501. [PubMed: 17046753]
- Zinck ND, Downie JW. Plasticity in the injured spinal cord: can we use it to advantage to reestablish effective bladder voiding and continence? *Prog Brain Res* 2006;152:147–162. [PubMed: 16198699]

Figure 1.

Immunohistochemistry with antibodies against PRV demonstrates the distribution of infected cells, 64 hours following delivery of virus to the left hemidiaphragm. At this time, both PhMNs and PRV-infected cervical interneurons were observed. No labeling was observed in the medulla at this time. Interneurons were seen bilaterally, in the dorsal horn around laminae II-V (A), and in laminae VII & X (B). Control experiments confirmed the authenticity of the transynaptic labeling of interneurons. Note that some putative interneurons were also observed immediately lateral (e.g. arrowhead in C) and medial to the tightly packed column of phrenic motoneurons (C). These results demonstrate the presence of a substantial population of cervical interneurons premotor to the phrenic nucleus. CC = central canal. Rostro-caudal orientation in

spinal cord sections is from right to left. Left to right sides of the spinal cord are from top to bottom in these images. Scale bar is 200 microns. Further details of these experiments are also described in Lane et al. (2008) *J Comp. Neurol.* 511(5): 692

Figure 2.

Longitudinal sections were taken through the cervical spinal cord 2 weeks following Miniruby injection. Note that injection of Miniruby in the region of the VRC resulted in some retrograde labeling of a separate population of interneurons that may innervate the region of the VRC (interneurons with ascending projections; **A**). Dual anterograde and retrograde tracing with Miniruby (VRC) and PRV152 (applied to the diaphragm) demonstrates bilateral bulbospinal projections (red) toward the soma and dendrites of PRV-positive (green) pre-phrenic interneurons (**B, C**) and phrenic motoneurons (**D**). Scale bars are 50 microns (**A, B** and **D**) and 25 microns (**C**). Rostro-caudal orientation is from top to bottom. Confocal microscopy was used to confirm the proximity of Miniruby positive VRC fibers with PRV labeled cells and a 3-D reconstruction made (**C**).

Figure 3.

Longitudinal sections of the cervical spinal cord following a lateral (left) C2 hemisection (C2HS). PRV positive interneurons were observed 2 weeks following SCI (A, B). While there was no detectable change in pattern of PRV labeling in either the PhMN pool (C) or the general distribution of cervical interneurons, the number of labeled cells decreased. CC = central canal. Rostro-caudal orientation in spinal cord sections is from right to left. Scale bar is 200 microns.

Figure 4.

Longitudinal sections of the cervical spinal cord showing retrogradely PRV-labeled pre-phrenic interneurons (**A**, **C**) and phrenic motoneurons (**B**, **D**), 2 weeks following C2 hemisection (C2HS). Anterogradely labeled projections from the VRC (red) ipsi- and contralateral to injury are seen in close association with the soma and dendrites of all cell populations. Qualitative analysis suggests that both phrenic motoneurons and pre-phrenic interneurons are more closely associated with projections from the contralateral VRC (**C**, **D**). Note also, the apparent reduction the extent of VRC input to PRV-infected cells relative to the uninjured rat spinal cord (Figure 2). Scale bar is 50 microns.

Figure 5.

Electrophysiological recording from the phrenic nerve ($\int \text{Phr}$) and a cervical interneuron (f_{Unit}) during ventilation with normal air (i) and hypoxic treatment (ii; $\text{FIO}_2=13.3$ for 3 minutes). These results demonstrate that hypoxia can differentially influence activity of cervical spinal cord interneurons (A). An expanded trace from A before (i) and after (ii) hypoxic treatment is shown in B. The interneuron exhibited phasic activity relative to the integrated phrenic nerve activity (Bi). Furthermore, hypoxia induced excitation was observed in this interneuron. $\int \text{Phr}$: integrated phrenic activity; f_{Unit} : discharge frequency of the spinal cord unit.