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Review

Pre-phrenic interneurons: Characterization and role in phrenic pattern formation and respiratory recovery following spinal cord injury

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ABSTRACT

The phrenic motor system receives excitatory inspiratory bulbospinal drive from inspiratory pre-motor neurons in the rostral ventral respiratory group and descending inhibition from bulbospinal Bötzinger complex units in the brainstem. While phrenic motoneurons have been extensively studied, a thorough understanding of the role of pre-phrenic interneurons in respiratory pattern formation is only beginning to emerge. Pre-phrenic interneurons are located at upper cervical spinal cord levels, as well as within and around the phrenic nucleus at mid-cervical levels. We speculate they may be involved in polysynaptic bulbospinal relays to phrenic motoneurons and/or may operate independently to modulate respiratory motor outputs. Additionally, pre-phrenic interneurons may serve as a neuroanatomic substrate for a putative spinal respiratory rhythm/pattern generator. Lastly, pre-phrenic interneurons also appear to play an important role in respiratory recovery following spinal cord injury. These various roles subserved by pre-phrenic interneurons are reviewed and discussed.

1. Anatomical organization

Respiratory interneurons projecting to phrenic motoneurons are typically found at the level of the cell they supply (Lane et al., 2008a; Lois et al., 2009; Qiu et al., 2010), but may also be found in the upper cervical region (Hoskin et al., 1988; Douse et al., 1992; Nakazono and Aoki, 1994; Nonaka and Miller, 1991; Qin et al., 2002a, b). As revealed by transsynaptic tracing (see Card and Enquist, 1999), they are located throughout all regions of the spinal gray matter and have been shown to make synaptic contact with respiratory motoneurons in the ventral horn in several species (Dobbins and Feldman, 1994; Billig et al., 1999; Yates et al., 1999; Billig et al., 2000, 2001; Gaytán et al., 2002; Lane et al., 2008a,b2009a,b,; Lois et al., 2009; Qiu et al., 2010).

There are several locations of pre-phrenic interneurons. These include upper and mid cervical spinal cord gray matter (ventral and dorsal horns), around the central canal, and within the phrenic nucleus. That retrograde transsynaptic labeling failed to reveal pre-phrenic interneurons in upper cervical levels suggests that these recorded respiratory-related neurons are not well-connected to the phrenic motoneuron pool. However, retrograde C-terminal tetanus toxin peptide labeling reveals a cluster of interneurons dorsolateral to the phrenic motoneuron pool, heaviest at C4 with some at C3 spinal cord levels, followed by a large gap without labeled cells, and finally labeled interneurons at C1-C2 levels of the spinal cord. Moreover, pre-phrenic interneurons appear to receive projections from ipsi- and contralateral rostral ventral respiratory group (rVRG) and may in turn project to 1) ipsilateral phrenic motoneurons, 2) contralateral phrenic motoneurons, or 3) bilateral phrenic motoneurons. This redundancy may prove beneficial in respiratory recovery following spinal cord injury-induced interruption of bulbospinal respiratory pathways (Ghali and Marchenko, 2015; Ghali, 2017b).

Pre-phrenic interneurons located at the phrenic nucleus levels are clustered in laminae VII and IX, in contrast to those located at upper cervical levels (*see below*), which are found in laminae VII and X around the central canal of the spinal cord (Dobbins and Feldman, 1994). Topical application of pseudorabies virus (PRV)-Bartha onto one side of the diaphragm revealed labeling of ipsilateral phrenic motoneurons at 24 h (Lane et al., 2008a,). At the 64 h time stamp, pre-phrenic interneurons were labeled bilaterally caudal to C2, but not at upper cervical levels, primarily in laminae VII and X, with a few in the dorsal horns labeled both ipsi- and contralaterally. Interestingly, interneurons which were in greater proximity to the phrenic motoneuron pool (just medial and lateral) were labeled at a later time (72 h) and the investigators of that study remain guarded in speculating as to the nature of their identity.

Application of two retrograde tracers fluorescing at different wavelengths (PRV-Bartha 152 and PRV-Bartha 164) to alternate sides of the diaphragm revealed bilateral labeling of second-order pre-phrenic

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interneurons at the level of the phrenic nucleus (Lane et al., 2008a,). Additionally, a subset of pre-phrenic interneurons co-labeled with tracers applied to left and right hemidiaphragms, suggesting that some of these cells may simultaneously regulate and couple left and right phrenic motoneuron pools across the midline (Lane et al., 2008a,). These may be an important substrate for recovery of crossed phrenic activity following high cervical hemisection (Ghali and Marchenko, 2015; Ghali, 2017a, b). To delineate supraspinal input to double-labeled pre-phrenic interneurons, inspiratory-related neurons were recorded in VRG, and injections of biotinylated dextran amine tracer were performed, as well as retrograde labeling of phrenic motoneurons using ipsilateral intra-diaphragmatic topical application of PRV (Lane et al., 2009a, b, c). Bulbospinal projections were observed bilaterally to phrenic motoneurons, as well as to pre-phrenic interneurons in laminae X (surrounding the central canal of the spinal cord). An analogous set of experiments revealed the same organization of pre-intercostal interneurons (Lane et al., 2008a). Finally, some pre-respiratory interneurons appear to supply both phrenic and intercostal MN pools simultaneously.

Retrograde transsynaptic labeling of the phrenic motor system with wheat germ agglutinin conjugated to horseradish peroxidase (WGA-HRP) and WGA-Alexa injected into the diaphragm labeled phrenic motoneurons and rVRG neurons bilaterally, but failed to label cervical propriospinal interneurons (Moreno et al., 1992; Buttry and Goshgarian, 2015), connectivity of rVRG with cervical propriospinal pre-phrenic interneurons remained unclear. Physiological studies, however, clearly demonstrated that respiratory-related interneurons in the cervical (Bellingham and Lipski, 1990) and thoracic (Kirkwood et al., 1988) spinal cord receive supraspinal inspiratory drive, as well as inputs from vestibular areas (Anker et al., 2006). Moreover, pre-phrenic interneurons labeled by topical application of PRV to the diaphragm were surrounded by VRG axons labeled by iontophoretically-applied Miniruby, via electrophysiological identification using multiunit recordings (see Fig. 9, C and D of Lane et al., 2008a). These data support a model whereby pre-phrenic interneurons receive descending projections and may serve as a relay interposed between medullary bulbospinal units and phrenic motoneurons.

2. Electrophysiological studies

2.1. Mid-cervical pre-phrenic interneurons

Respiratory-related interneurons fire cyclically in phase with respiration, as determined from the tracheal pressure or respiratory-related peripheral neurogram or myogram activity. They may be predominantly inspiratory or expiratory (Cleland and Getting, 1993; Grelot et al., 1993; Iscoe and Duffin, 1996; Lane et al., 2009a,b; Streeter et al., 2017) and might serve to inhibit or excite phrenic (or other respiratory-related) motoneurons via direct or indirect interactions (Aminoff and Sears, 1971; Bellingham and Lipski, 1990; Kirkwood et al., 1993; Bellingham, 1999; Streeter et al., 2017). In a study by Streeter and colleagues (2017), cervical pre-phrenic interneurons with excitatory and inhibitory phenotypes were identified, with the former exhibiting principally tonic or inspiratory discharge patterns and the latter exhibiting a principally expiratory pattern (Streeter et al., 2017). In addition to receiving supraspinal inputs (Hoskin and Duffin, 1987a, b; Mateika and Duffin, 1989), these interneurons may receive afferent feedback from the panoply of respiratory-related networks (Goshgarian and Roubal, 1986; Dawkins et al., 1992; Duffin et al., 1994; Iscoe and Duffin, 1996; Chandler et al., 1998; Bellingham, 1999; Iscoe, 2000).

The first functional evidence for spinal respiratory interneurons was demonstrated in decorticate rabbits (Palisses et al., 1989). These were recorded in C4-C6 spinal cord, exhibited respiratory-related modulation, and were positively identified by failure to respond to antidromic activation of phrenic nerve. Interneurons were classified as inspiratory or expiratory depending on their firing relative to respiratory phases. Simultaneous recording of bulbospinal inspiratory neurons showed that their firing did not coincide with that of the interneurons, suggesting a more complex role for the latter than simple relays. They were located dorsomedially to phrenic motoneurons and extended slightly more rostral and less caudal than the phrenic motoneuron column. These results were later corroborated in 1993 in pentobarbitone-anesthetized cats, where C5 expiratory interneurons were identified by failure to respond to antidromic activation or show a unitary waveform upon spike-triggered averaging of phrenic nerve discharge (Douse and Duffin, 1993). They were found to be located dorsomedial to phrenic motoneurons and project their axons in the ventrolateral funiculus to ipsilateral C6 phrenic motoneurons. Their firing patterns were characterized as constant, augmenting, or decrementing. A population of putative pre-phrenic interneurons were also observed at the mid-cervical spinal cord in the adult rat. These interneurons exhibited respiratory-related firing and responded to respiratory stimulus (i.e., hypoxia)(Lane et al., 2009a,b,; Sandhu et al., 2015; Streeter et al., 2017).

2.2. Upper cervical pre-phrenic interneurons

Upper cervical inspiratory neurons, originally identified in the cat (Aoki et al., 1980), send fibers toward intercostal motoneuron pools more so than phrenic nuclei (Aoki et al., 1980; Duffin and Hoskin, 1987; Lipski and Duffin, 1986; Hoskin et al., 1988; Douse et al., 1992; Nakazono and Aoki, 1994; Tian and Duffin, 1996a); in rats, Lipski et al. (1993) showed that these axons travel into upper thoracic levels. Tian and Duffin's cross-correlation studies in the decerebrate rat, however, failed to reveal monosynaptic connections between intercostal motoneurons, with either rVRG or C1/C2 inspiratory interneurons (Tian and Duffin, 1996a, b). In pentobarbitone-anesthetized rats, cross-correlation analysis between upper cervical inspiratory neurons with phrenic bursting revealed no monosynaptic connections, although approximately 4/5 of these units were antidromically-activated by stimulation at the C7/C8 border. In the same study, the few cells traced intracellularly with neurobiotin sent collaterals near the phrenic nucleus (Lipski et al., 1993) and continued into upper thoracic levels. In the decerebrate rat, however, cross-correlation analysis revealed monosynaptic connections between C1/C2 inspiratory units with rVRG units rostrally (Tian and Duffin, 1996a) and with phrenic motoneurons caudally (Tian and Duffin, 1996b) (Fig. 1). In this light, it has been shown that monosynaptic projections controlling respiration become dominant with maturation relative to polysynaptic pathways (Juvin and Morin, 2005). Thus, use of pentobarbital in Lipski et al.'s (1993) study may have preferentially silenced a weak polysynaptic bulbophrenic pathway, which in the decerebrate rat remains active, permitting cross-correlation analysis to identify monosynaptic connectivity of putative relay inspiratory units with both rVRG (Tian and Duffin, 1996a) and phrenic motoneurons (Tian and Duffin, 1996b).

3. Functional significance of respiratory-related interneurons

3.1. Overview

Spinal interneurons with respiratory-related activity have been proposed to act as relays in a polysynaptic supraspinal-interneuron-motoneuron projection to the phrenic and intercostal motor systems (Palisses and Viala, 1987; Palisses et al., 1989; Hayashi et al., 2003; Anker et al., 2006; Butler, 2007; Lane et al., 2008a,). Additionally, respiratory interneurons may be involved in mediating reflexes (Speck and Revelette, 1978; Downman, 1955; Eccles et al., 1962; Douse et al., 1992; Bellingham, 1999), such as the phrenic-phrenic reflex (Duron et al., 1976) or may contribute to neuro-computational processing by integrating peripheral sensory information, motoneuronal activity, and supraspinal drive (Jankowska and Hammar, 2002; Lois et al., 2009; Billig et al., 2000; Lane et al., 2008a), aiding in the smooth recruitment of motor units (Renshaw, 1941) according to the Henneman size

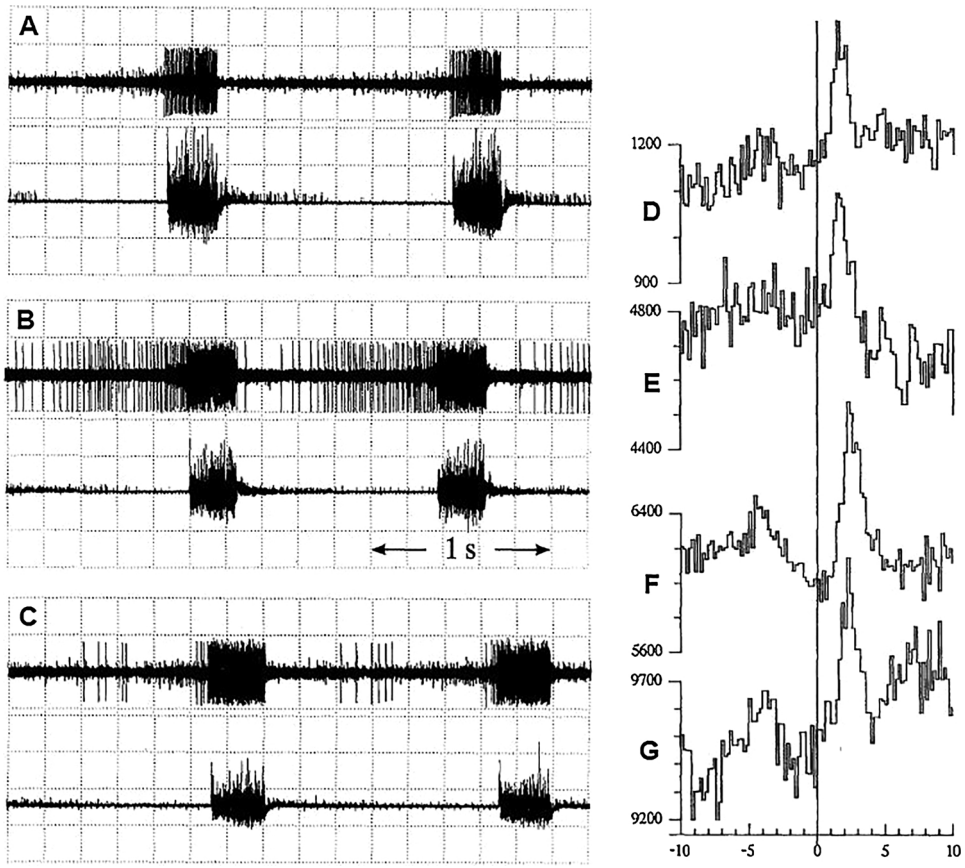

Fig. 1. Upper cervical inspiratory neuron discharge patterns and cross-correlation with ipsilateral phrenic nerve. A: A neuron with discharge confined to inspiration. B: neuron with tonic activity during the expiratory phase. C neuron with weak tonic discharge during expiration. Top traces in A–C: upper cervical inspiratory neuron activity; lower traces in A–C phrenic nerve discharge. The grid in A–C marks intervals of 0.2 s. D–G: Cross-correlation histograms computed between the discharge of upper cervical inspiratory neurons and the ipsilateral phrenic nerve. Narrow peaks at short latencies were interpreted as evidence for monosynaptic connections. Bin width 0.2 ms; vertical axes, counts per bin. Reprinted with permission from Figs. 1 and 3 of Tian and Duffin (1996b).

principle (Henneman et al., 1965). Furthermore, it has been suggested that while bulbospinal monosynaptic projections to phrenic motoneurons are extensive, they are not sufficient to account for the depolarization of the entire phrenic motoneuron pool (and cervical pre-phrenic interneurons have been suggested to play a critical role in modulating coordinate discharge within phrenic motoneuron pools and across the midline (Davies et al., 1985 Jankowska and Hammar, 2002; Lois et al., 2009).

A recent report further demonstrated that acute intermittent hypoxia enhances functional connectivity in a subset of mid-cervical spinal interneurons, which may putatively play a role in initiation of neuroplasticity of the phrenic motor output during and/or after hypoxic stress (Streeter et al., 2017). Exposure of animals to acute intermittent hypoxia induces a phenomenon known as phrenic long term facilitation, whereby amplitude of phrenic nerve discharge is increased in a sustained fashion following exposure to brief episodes of hypoxia (Fuller and Mitchell, 2017). Acute intermittent hypoxia holds significant therapeutic promise for enhancing respiratory recovery following spinal cord injury (Gonzalez-Rothi et al., 2015) and the network of cervical pre-phrenic interneurons represents a critical intermediary in shaping the phrenic neuroplastic response to this treatment.

The hypoxic stimulus may be relayed to cervical pre-phrenic interneurons from supraspinal respiratory (Fedorko et al., 1983; Lane et al., 2008a) or sympathetic centers (Guyenet, 2000) or may be detected by the interneurons proper, as evidenced by the demonstration of oxygen sensing cells within the spinal cord(Wilson et al., 2015). Hypoxia is well-characterized as a potent stimulus for the release of serotonin (Kinkead et al., 2001) and latent polysynaptic bulbophrenic pathways may be activated by hypoxia through serotonergic mechanisms (Li and Zhuo, 1998; Mitchell et al., 1992; Ling et al., 1994). Such polysynaptic relays may figure prominently as a critical neuroanatomic substrate in respiratory recovery and crossed phrenic activity following high cervical spinal cord injury (Ghali and Marchenko, 2015). One pathway characterized as mediating hypoxia-induced phrenic long term facilitation involves serotonergic activation of mitogen-activated protein kinase and extracellular-regulated kinase signaling via TrkB and protein kinase C (Devinney et al., 2015; Dale et al., 2017). Further studies are necessary to more fully elucidate the role of cervical pre-phrenic interneurons in mediating phrenic long term facilitation in response to acute intermittent hypoxia and molecular mechanisms responsible for the same.

A functional importance for respiratory-related spinal interneurons is suggested by the finding that rhythmic bursting is often observed in phrenic nerve after spinalization (Coglianese et al., 1977; Aoki et al., 1978; Viala and Freton, 1983; Ghali and Marchenko, 2016). Spontaneous respiratory rhythm generation following spinalization was demonstrated in cats and dogs in the 1970's. Recently, we demonstrated that spontaneous patterns of discharge may be observed in phrenic nerve recordings following C1 transection (Ghali and Marchenko, 2016), as well as in response to asphyxia (Ghali and Marchenko, 2016) or disinhibition of C1-C2 spinal cord via topical application (Ghali and Marchenko, 2016) or microinjection of GABAzine and strychnine. Thus, a local circuit must be extant and operative to explain these observations; if an interneuronal pool did not exist and phrenic motoneurons could be driven only by descending bulbospinal monosynaptic projections, then phrenic bursting would not be observed following spinalization (nor could afferent stimulation trigger a phrenic motor response) either spontaneously or following experimental provocations. For these reasons, some have suggested the existence of a spinal respiratory central pattern generator that may function without supraspinal descending inputs (Zaki Ghali, 2013; Ghali and Marchenko, 2016); the substrate for the network circuitry would thus involve propriospinal interneurons (Coglianese et al., 1977; Aoki et al., 1980; Palisses et al., 1989; Alilain et al., 2008, 2011), though further studies are necessary to more precisely characterize network properties.

A group of neurons in the upper cervical region extending from the

medullospinal junction to the C2 spinal segment and 100–300 μm deep to the ventral surface has recently been shown by optical imaging of voltage-sensitive dye in a brainstem-spinal cord *en bloc* preparation of the newborn rat to exhibit endogenous respiratory-related activity (including pre-I and I discharge) following cervical transection (Oku et al., 2008). This may represent a neonatal spinal respiratory generator, which may be disinhibited pharmacologically in the adult (Zaki Ghali, 2013; Ghali and Marchenko, 2016). Interestingly, Cregg et al. (2017) recently reported that there may be another latent propriospinal network caudal to the phrenic motoneuron pool, which can trigger rhythmic phrenic bursting in the presence of inhibitory neurotransmitter antagonist following C1 transection in neonatal mice. (Cregg et al., 2017). This type of spinal cord derived phrenic bursting can be also evoked in both adult mouse and rat model after blockade of glycine and GABA receptors (Cregg et al., 2017).

3.2. Role of inhibitory interneurons in phrenic pattern formation

The phrenic nucleus receives $GABA_A$ergic inhibition during inspiration. For example, unilateral microinjections of gabazine ($GABA_A$ receptor antagonist) within the phrenic nucleus result in increased phrenic bursting amplitude throughout all respiratory phases (inspiration, post-inspiration and expiration) in decerebrate rats (Marchenko and Rogers, 2009). Reciprocally, microinjections of $GABA_A$ and $GABA_B$ agonists decreased phrenic bursting amplitude only during inspiration in the same model (Chitravanshi and Sapru, 1999), demonstrating fast inhibitory synaptic transmission plays an important role in phrenic patternogenesis.

Phrenic inhibition was also evaluated in the neonatal rat brainstem-spinal cord *en bloc* preparation. Responses to intracellular injections of current were shown to be greater during both phases of expiration (post-inspiration and E2 expiration) than inspiration, possibly as a result of shunting inhibition during inspiration (Parkis et al., 1999). This study also provided the evidence that there must be a source of inhibition to phrenic motoneurons caudal to the border between BötzC and pre-BötzC (Parkis et al., 1999). At the individual cell level, some phrenic motoneurons show increased activation following blockade of inhibition (Marchenko and Rogers, 2007b); bicuculline caused increased activity of individual phrenic motoneurons during all phases of respiration, while the facilitative/disinhibitory effects of strychnine were restricted to post-inspiration. It is important to note that the effects of $GABA_A$ergic transmission in vitro in neonatal rats may mediate excitatory responses (Su and Chai, 1998), as a consequence of differential expression of transporters mediating cellular import and export of chloride ions (Delpire, 2000; Russell, 2000).

Local microinjection restricts the effects of pharmacological blockade to postsynaptic receptors of a specific subpopulation of neurons of interest, but does not discriminate between blockade of distant projection neurons and local interneurons. Thus, gabazine and strychnine microinjections will block all descending bulbospinal and local intraspinal $GABA_A$- and glycinergic signaling and will not inform whether the former, latter, or both are operant. Bulbospinal GABAergic neurons providing inhibition to phrenic motoneurons during inspiration were demonstrated in the in vitro neonatal rat brainstem-spinal cord preparation (Parkis et al., 1999). Treatment with nicotinic acetylcholine receptor antagonists in C4 ventral horn did not eliminate this inhibition, confirming that the inspiratory inhibition was not mediated by phrenic motoneuron axon collaterals exciting recurrent inhibitory Renshaw cells. Bulbospinal GABAergic units were also demonstrated neuroanatomically located in the rVRG and the Bötzinger complex (Ellenberger, 1999).

Several studies have provided evidence for involvement of Renshaw cells in local inhibitory modulation of phrenic motor output. For example, Renshaw cells were excited and inhibitory postsynaptic potentials were induced in phrenic motoneurons by stimulation of C5 ventral root in anesthetized cats, functionally demonstrating the existence of local intraspinal inhibitory transmission operant independently of descending inhibition (Hilaire et al., 1986). This was also demonstrated in deafferented cats, where antidromic stimulation of C5 caused inhibition of phrenic motoneurons and activation of Renshaw cells. Thus, evidence exists for both descending bulbospinal and local intraspinal inhibitory transmission. Some interneurons in ventral horn of cervical spinal cord have been shown to co-label for Glutamate decarboxylase (GAD67) and Glycine transporter 2 (GLYT2), demonstrating the existence of combined GABA/glycinergic interneurons and adding further complexity to the precise nature of local inhibitory circuitry (Mackie et al., 2003).

The role of local intraspinal GABAergic pre-phrenic interneurons in respiratory pattern formation has been confirmed by our group (Marchenko et al., 2015). Given that pharmacological antagonism with gabazine/bicuculline causes blockade of both descending and local fast inhibitory mechanism, we opted to use RNA interference in order to effect a local downregulation of the translation of both isoforms (65 and 67 kDa) of the GABA-synthesizing enzyme glutamate decarboxylase using unilateral phrenic nucleus microinjections of small interfering RNA (siRNA) targeting the same. This treatment resulted in increased activity in phrenic discharge ipsilateral to microinjections during the second half of inspiration and post-I and E2 phases of expiration, demonstrating a role for local mid-cervical pre-phrenic GABAergic interneurons in both phasic and tonic control of phrenic pattern formation (Fig. 2). Corroboratively, a downregulation in the number of GABAergic cells was shown in response to treatment with anti-GAD65/67 siRNA (Fig. 3). This data provides evidence for the existence of respiratory-related pre-phrenic interneurons exhibiting a GABAergic phenotype and demonstrates an important role for local intraspinal fast inhibitory mechanisms in phrenic pattern formation. We suggest that local GABAergic pre-phrenic interneurons are more critical for providing inhibition of phrenic motoneurons during the inspiratory phase, whereas descending units provide inhibition throughout all respiratory phases.

3.3. Role of interneurons in respiratory recovery following spinal cord injury

An appreciation for the capacity of the spinal cord to undergo extensive functional neuroplasticity following injury has increased in recent years, as well as an understanding of the significance and ramifications of the same (Fouad and Tse, 2008; Lane et al., 2008b; Darian-Smith, 2009). Contribution of interneurons to neuroplasticity has been demonstrated across several studies in a variety of settings (Krassioukov et al., 2002; de Groat and Yoshimura, 2006d; Llewellyn-Smith et al., 2006; Rabchevsky, 2006; Zinck and Downie, 2006; Hou et al., 2008a, b). This has been well-demonstrated in the locomotor system, wherein long and short propriospinal interneurons serve differential roles in motor recovery following spinal cord injury. The former traverse multiple spinal cord segments and are thus suitably positioned to provide a substrate for functional recovery of the locomotor generator (Bareyre et al., 2004; Courtine et al., 2008), whereas the latter can regulate and modulate motoneuronal activity and may subsume novel functional roles contributing to recovery (Jankowska, 1985).

Following spinal cord injury, significant reorganization of locoregional circuitry also occurs in the respiratory network, fundamentally altering its microanatomy and neurocytoarchitecture compared to the native uninjured state (Dimitrijevic et al., 1997). For example, following C2 hemisection, the number of PRV-labeled interneurons relative to phrenic motoneurons is reduced in the rat (Lane et al., 2009a). At 2 weeks post-injury, there is a reduction of interneurons in *contralateral* laminae VII and X and, at 12 weeks, the reduction is observed *bilaterally*. Notable is the relatively unaltered number of dorsal horn interneurons with respect to phrenic motoneurons, underscoring potential differential regulation and functionality of dorsal versus ventral horn interneuronal pools following injury. Moreover, Zholudeva et al. (2017) demonstrated that a specific type of V2a interneuron is recruited following high cervical spinal cord injury in the mouse model (Zholudeva et al., 2017).

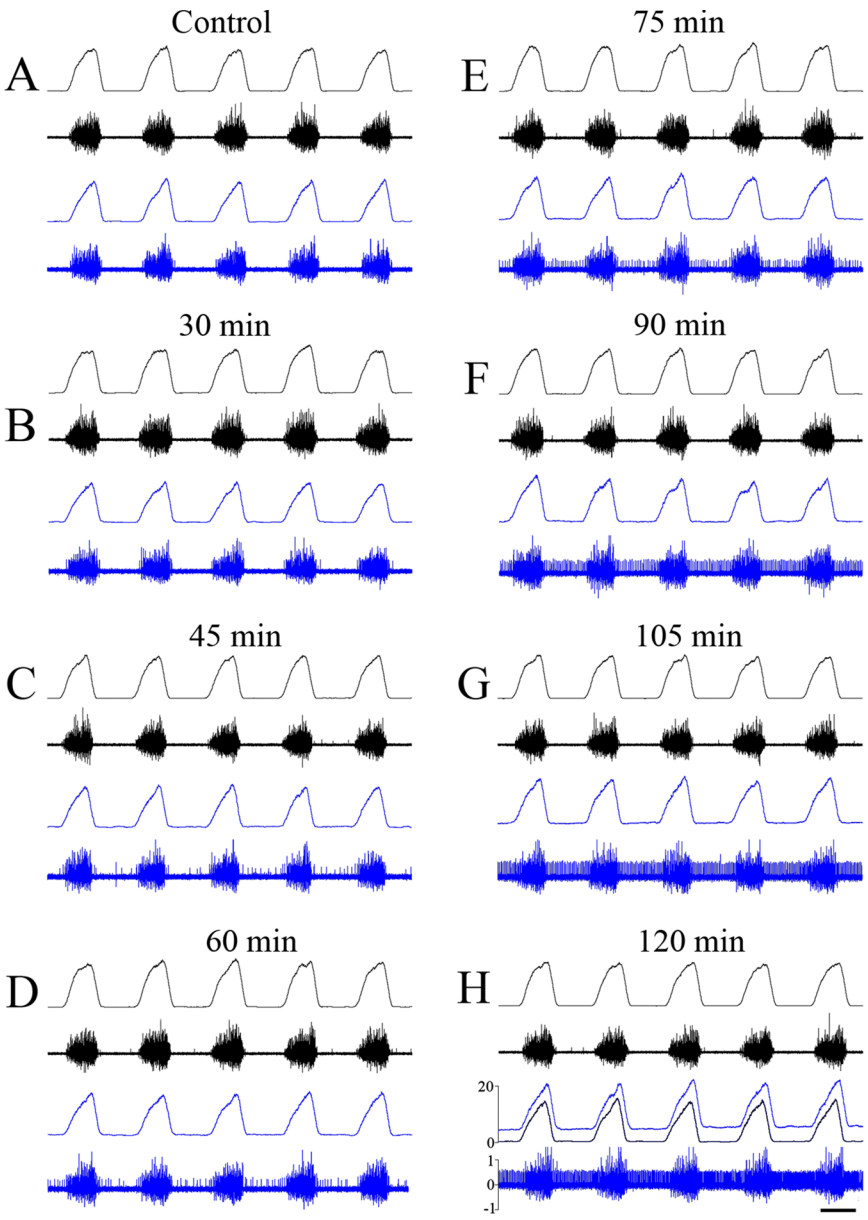

Fig. 2. Phrenic nerve activity dynamics during anti-GAD65/67 siRNA microinjecton into the phrenic nucleus. Raw and integrated phrenic nerve activity at different time points relative to the beginning of anti-GAD65/67 siRNA microinjections. A: before microinjections. B: 30 min C: 45 min. D: 60 min. E: 75 min. F: 90 min. G: 105 min. H: 120 min, overlapping of two integrated phrenic traces before and after 2 h of anti-GAD65/67 siRNA microinjectons. Scales for integrated phrenic activity are shown in arbitrary units and for raw activity in mV (x10^5 amplification). Upper two subpanels: raw and integrated phrenic nerve activity of intact side. Lower two subpanels: raw and integrated phrenic nerve activity on the side of anti-GAD65/67 siRNA microinjection. Modified with permission from Fig. 3 of Marchenko et al. (2015).

Functional and anatomical changes within pre-phrenic interneuronal circuitry may putatively be involved in respiratory recovery following spinal cord injury. Recruitment and incorporation of excitatory cervical interneurons may contribute to recovery of phrenic output ipsilateral to a C2 hemisection, contusion, or analogous injury (Lane et al., 2009a, b,; Sandhu et al., 2009), as occurs in other motor systems (Bareyre et al., 2004; Courtine et al., 2008, 2009). Restoration of function may be promoted by the recruitment of long (Bareyre et al., 2004; Courtine et al., 2008) or short (Jankowska, 1985) propriospinal interneurons. C1-C2 pre-phrenic interneuron recruitment following hemisective spinal cord injury and phrenic motor network coaptation of these cells may result from genesis of polysynaptic relays to the phrenic motor nucleus. For example, following a C1 (Hayashi et al., 2003) or C2 (Gonzalez-Rothi et al., 2017) hemisection, short-term potentiation could be observed several seconds after direct or epidural high-frequency stimulation of the ipsilateral lateral funiculus, suggesting the activation of a pathway involving interposed interneurons (Hayashi et al., 2003; Gonzalez-Rothi et al., 2017). Alternatively, reduction of inhibitory pre-phrenic interneurons may be able to increase the excitability of phrenic motoneurons through disinhibitory effect.

As contrasted with spinal-intact animals, where PRV fails to retrogradely label C1–2 respiratory-related interneurons, retrograde transsynaptic labeling via diaphragmatic application of PRV extensively labeled C1-C2 pre-phrenic interneurons bilaterally following C2 HSx (Lane et al., 2009a). Pre-phrenic interneurons projecting to phrenic nuclei bilaterally (Lane et al., 2008a,) may serve to synchronize respiratory motor outputs, by virtue of left-right coupling of phrenic nuclear output (Sandhu et al., 2009), as well as top-down coupling with intercostal motor nuclei, and may contribute to functional recovery of spontaneous crossed phrenic activity (Ghali and Marchenko, 2015). Such anatomical and functional network changes may underlie robust recovery of phrenic motor output following cervical spinal cord injury. For instance, following supraphreninuclear hemisection, there is recovery of output of the phrenic nucleus ipsilateral to injury, as well as increased amplitude of phrenic output contralateral to both C1 (Ghali and Marchenko, 2015) and C2 hemisective injury (Golder et al., 2003; Fuller et al., 2006).

Greater and more rapid recovery of phrenic nerve activity ipsilateral to a C1 (Ghali and Marchenko, 2015; Ghali, 2017a, b, see Tables 1 and 2), compared to a C2 hemisection, may putatively result from sparing of a pool of C1-C2 pre-phrenic interneurons; of rostrally-located units anatomically and of more caudally-located units by limiting of descending edema from hemisective injury. These interneurons may either augment ipsilateral

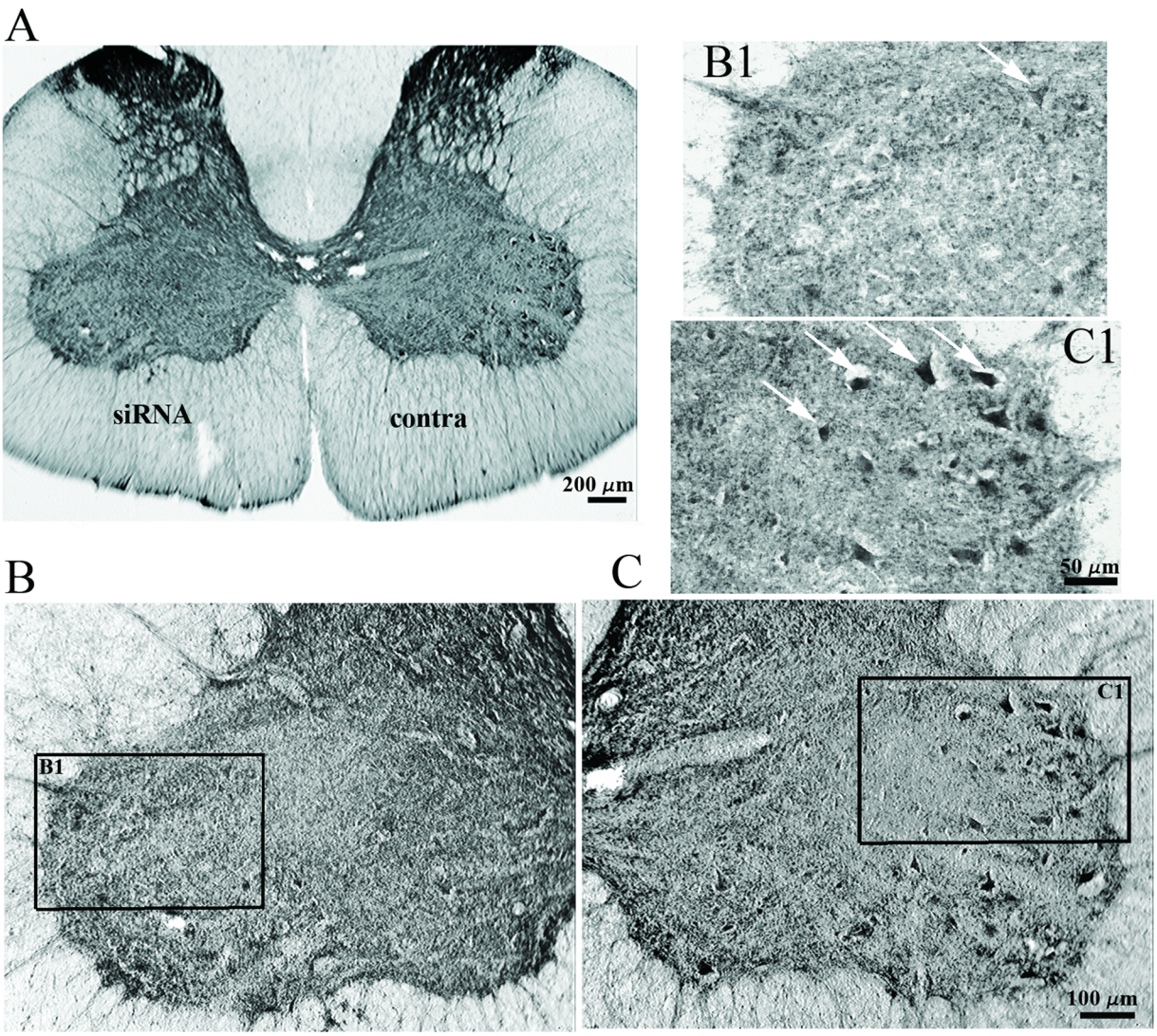

Fig. 3. Distribution of GAD65/67-containing spinal interneurons after unilateral anti-GAD65/67 siRNA microinjection into the C4 ventral horn. *A:* C4 transverse section following a 2 h unilateral anti-GAD65/67 siRNA block ('siRNA') vs. contralateral side ('contra'). *B-C:* magnified view of ventral horn on ipsi ('*B*') and contralateral side ('*C*') showing clearly labeled GAD-positive neurons (white arrows indicate some in focal plane on high power magnification panels '*B1*' and '*C1*'). Modified with permission from Fig. 6 of Marchenko et al. (2015).

phrenic nerve activity by way of crossed polysynaptic bulbophrenic relays, via descending tonic excitation independent of bulbar inputs, or via both mechanisms. These hypotheses await empirical verification.

4. Perspectives and Significance

Thus, the phrenic motor network can be conceptualized as driven by direct descending monosynaptic bulbophrenic projections, polysynaptic projections interfacing with a network of cervical propriospinal pre-phrenic interneurons, along with a bulbar-independent network of pre-phrenic interneurons. That the phrenic motor output is organized in this manner contemporaneously illumines and mystifies network dynamics. How would phrenic motor output differ without a significant contribution by a cervical pre-phrenic interneuronal interface between descending units and ultimate effector motor nuclei? We may in general terms posit a few possibilites as to the possible role served by pre-phrenic interneurons. The simultaneous presence of excitatory and inhibitory pre-phrenic interneurons with inspiratory and expiratory phase preference allows for the capacity for extremely precise fine tuning of respiratory pattern formation. Asphyxia induces a decrementing rhythmic activity in phrenic nerve discharge in spinalized animals highly akin to gasping generated in spinal-intact animals; thus, native spinal circuitry generates a rudimentary rhythmic activity and descending bulbar projections are responsible for generation of an augmenting respiratory pattern. Pre-phrenic interneurons may also play a role in gating and determining the gain in response to direct monosynaptic, as well as indirect polysynaptic, descending inputs. The presence of pre-phrenic interneurons, as well as descending monosynaptic bulbophrenic units, projecting simultaneously to ipsilateral and contralateral phrenic motor nuclei permits a high fidelity of phrenic motor neuron coupling across the midline. Previous data have demonstrated a powerful contribution of GABAergic inhibition, originating supra- and intraspinally, in coupling and synchronizing high frequency oscillations (Marchenko and Rogers, 2009) (the direct contribution of local GABAergic inhibition to high frequency oscillations remains to be determined). Furthermore, cervical interneuronal neuroplasticity arms the respiratory network with the capacity for adapation following spinal cord injury.

5. Conclusion

Cervical interneurons have been extensively characterized anatomically and physiologically. The body of literature suggests these cells may play a role in phrenic pattern formation and recovery following

spinal cord injury. There may exist functionally-distinct interneuronal populations, one serving as a supraspinal relay, possibly functioning to amplify descending drive (Hayashi et al., 2003), and the other acting independently of brainstem input, possibly driven by other interneurons or recurrent phrenic motoneuron collaterals and forming the neuroanatomical substrate for a putative spinal respiratory rhythm generator. Clearly, the interneuron story is far from complete and it will be interesting to see how it unfolds as investigators make strides into assigning a more specific role to this "black-box" of respiratory neurophysiology. Further studies are necessary to more precisely characterize the role of pre-phrenic interneurons in respiratory pattern formation and recovery following spinal cord injury.

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Conflicts of interest

None.

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