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Neuromuscular adaptations to respiratory muscle inactivity

Carlos B. Mantilla and Gary C. Sieck

Departments of Physiology & Biomedical Engineering and Anesthesiology, Mayo Clinic, Rochester, MN 55905

Abstract

Cervical spinal cord injury results in significant functional impairment. It is important to understand the neuroplasticity in response to inactivity of respiratory muscles in order to prevent any associated effects that limit functional recovery. Recent studies have examined the mechanisms involved in inactivity-induced neuroplasticity of diaphragm motor units. Both spinal hemisection at C₂ (C2HS) and tetrodotoxin (TTX)-induced phrenic nerve blockade result in diaphragm paralysis and inactivity of axon terminals. However, phrenic motoneurons are inactive with C2HS but remain active after TTX. Diaphragm muscle fibers ipsilateral to C2HS display minimal changes post-injury. Neuromuscular transmission is enhanced following C2HS but impaired following TTX. Synaptic vesicle pool size at diaphragm neuromuscular junctions increases after C2HS, but decreases after TTX. Thus, inactivity-induced neuromuscular plasticity reflects specific adaptations that depend on inactivity at the motoneuron rather than at axon terminals or muscle fibers. These results indicate that neuromuscular transmission and functional properties of DIAM fibers can be maintained after spinal cord injury, providing a substrate for functional recovery and/or specific therapeutic approaches such as phrenic pacing.

Keywords

Fiber Type; Inactivation; Motor Unit; Phrenic; Plasticity; Transmission

1. Introduction

Inactivity of respiratory muscles may result from defects in the activation of respiratory pathways (e.g., after an upper cervical spinal cord injury), impaired neuromuscular transmission or primary muscle disorders. Any of these may lead to life-threatening deficits in the ability to sustain ventilation. Many patients will require mechanical ventilation resulting in increased morbidity and mortality related to ongoing problems with pulmonary clearance, infections, and lung injury (Brown et al., 2006; Linn et al., 2000).

As the main inspiratory muscle in mammals, the diaphragm muscle is highly active, with a duty cycle of 30–40% compared to ~1–2% for the soleus and ~15% for extensor digitorum longus muscles (Hensbergen & Kernell, 1997; Kong & Berger, 1986). Thus, the diaphragm may be particularly susceptible to inactivity or disuse. In the present review, we will focus on

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Corresponding Author: Carlos B. Mantilla, M.D., Ph.D., 4-184 W. Joseph, Saint Marys Hospital, Mayo Clinic, 200 First Street SW, Rochester, MN 55905, Tel: (507) 255-7481, Fax: (507) 255-7300, mantilla.carlos@mayo.edu.

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inactivity-induced adaptations displayed by diaphragm motor units (i.e., the motoneuron and the muscle fibers it innervates) and discuss mechanisms underlying such plasticity. These findings may be particularly important to the recovery of respiratory function in patients following cervical spinal cord injury reviewed in other sections of this Special Issue.

2. Models of respiratory inactivity

Various experimental models of disuse or inactivity have been used to examine the effects of inactivity on motor unit properties. The effects of muscle inactivity have been studied primarily in hindlimb muscles (Gundersen, 1985; Kotsias & Muchnik, 1987; Roy et al., 2007; Spector, 1985; St-Pierre & Gardiner, 1985; Zhong et al., 2007). In assessing the impact of inactivity, it is important to consider the prior activation history of a muscle. In hindlimb muscles, activation history is quite varied and difficult to quantify. In contrast, diaphragm activation is stereotypical in that, as the major inspiratory muscle, the diaphragm is continuously activated throughout life (Mantilla & Sieck, 2008b; Sieck & Fournier, 1989). In addition, models of muscle inactivity may differ in many respects including whether any residual muscle activity persists (Fournier et al., 1983). Importantly, innervation of the diaphragm muscle does not correlate with the anatomical divisions of sternal, costal and crural regions, but rather follows a ventrodorsal orientation with considerable overlap of different cervical segments within each region and without differences in fiber type distribution across regions (Fournier & Sieck, 1988; Sieck et al., 1983). Thus, it is unlikely that differences in respiratory-related activation occur across different diaphragm regions.

2.1. Cervical spinal cord injury

A well-established model of respiratory inactivity is the C2 spinal hemisection (C2HS) model (Figure 1). Phrenic motor neurons innervating the rat diaphragm muscle are located within lamina IX of cervical spinal cord segments C3–C5 (Prakash et al., 2000; Song et al., 2000), although some motoneurons may be present in the most rostral aspect of C6 (Goshgarian & Rafols, 1981). With a C2HS that includes the anterior and lateral columns (predominant descending bulbospinal pathways to the phrenic motor nucleus), phrenic nerve and diaphragm muscle activity completely disappear ipsilaterally (Fuller et al., 2002; Golder et al., 2003; Goshgarian et al., 1991; Miyata et al., 1995; Nantwi et al., 1999; Prakash et al., 1999; Zhan & Sieck, 1992), but rhythmic activity is restored over time either spontaneously (Fuller et al., 2006; Golder et al., 2001a; Golder et al., 2001b; Vinit et al., 2006) or as a result of increased drive or removal of inhibitory afferent feedback, e.g., following contralateral nerve injury (Goshgarian, 1981; Porter, 1895). Most of the literature regarding the effects of C2HS on diaphragm motor unit plasticity has been derived from rats; but the location and general distribution of phrenic motoneurons is highly conserved across mammals. Indeed, phrenic motoneurons are found at C4–C6 in cats (Webber et al., 1979), C5–C7 in ferrets (Yates et al., 1999), and C3–C5 in humans (Keswani & Hollinshead, 1955). Phrenic motor neurons receive rhythmic excitatory drive from premotor neurons located in the medulla (Ellenberger & Feldman, 1988; Feldman et al., 1985), namely the rostral ventral respiratory group in rats (Dobbins & Feldman, 1994; Ellenberger et al., 1990) or the ventral and dorsal respiratory groups and medial reticular formation in cats and ferrets (Feldman et al., 1985; Yates et al., 1999). Thus, similar mechanisms underlying neuroplasticity following an upper cervical spinal cord injury and functional recovery may be expected across species, highlighting the potential usefulness of animal models of C2HS in the study of respiratory recovery in humans, notwithstanding that the mechanisms and nature of spinal cord injury may differ.

Another important consideration relates to the experimental conditions under which functional recovery is evaluated. For example, many studies have employed an experimental condition that involves animals that are tracheostomized, vagotomized, paralyzed, and mechanically ventilated and phrenic nerve activity is recorded (Fuller et al., 2006; Fuller et al., 2002; Golder

et al., 2003; Golder et al., 2001a; Golder et al., 2001b; Vinit et al., 2006). Clearly, this is far from a “normal” physiological condition, and motor output is undoubtedly affected by these experimental manipulations (see comment by Sandhu et al., 2009). How this impacts functional recovery is unclear. Conversely, other studies, including our own, have employed phrenic nerve recordings (Miyata et al., 1995) or diaphragm electromyographic (EMG) recordings in anesthetized, spontaneously breathing animals (Goshgarian et al., 1991; Prakash et al., 1999; Zhan & Sieck, 1992). Certainly, anesthetics influence phrenic motor output and this may also influence the expression of functional recovery after spinal cord injury. Ideally, it would be best to assess functional recovery in unanesthetized, spontaneously breathing animals using chronically implanted electrodes (Dow et al., 2006; Sieck & Fournier, 1990; Trelease et al., 1982). We routinely verify the absence of ipsilateral diaphragm muscle EMG activity at the time of C2HS and at 3 days post-C2HS using pre-implanted diaphragm electrodes. In recent unpublished observations using this method, we documented the ability to obtain stable diaphragm EMG recordings for up to 60 days in adult rats, and found a progressive increase in the proportion of animals displaying spontaneous recovery of rhythmic diaphragm EMG activity ipsilateral to C2HS over time (see Sieck & Mantilla, 2009b). Only ~10% of unanesthetized animals displayed recovery at 7 days after C2HS, ~30% at 14 days after C2HS, and ~70% at 28 days after C2HS.

2.2. Phrenic motoneuron inactivity vs. diaphragm muscle paralysis

Interrupting the neural impulse along a phrenic nerve, either as a result of nerve injury (e.g., denervation – DNV) or conduction blockade induced by tetrodotoxin (TTX), results in ipsilateral diaphragm muscle paralysis. Both DNV and TTX result in complete diaphragm inactivation (Rowley et al., 2005; Zhan et al., 1997). These models differ only in that axoplasmic flow of nerve-derived or muscle-derived trophic substances is preserved after TTX, but interrupted after DNV. Axoplasmic flow of nerve-derived or muscle-derived trophic substances is also preserved with diaphragm muscle paralysis induced by C2HS. The TTX and C2HS models differ in that activity of phrenic motoneurons persists with TTX, while C2HS induces motoneuron inactivity, i.e., action potential generation is absent (or minimal) in phrenic motoneurons after C2HS but is present after TTX. Thus, the importance of trophic influences vs. motoneuron inactivity *per se* can be assessed by comparing neuromuscular adaptations under these different conditions.

3. Diaphragm muscle fiber adaptations to inactivity

Diaphragm DNV and/or TTX transiently (within the first week) increase the cross-sectional area of type I and IIa muscle fibers with little or no change in the cross-sectional area of type IIx and/or IIb fibers (Aravamudan et al., 2006; Geiger et al., 2003; Yellin, 1974; Zhan & Sieck, 1992). Subsequently, type IIx and/or IIb fibers display significant atrophy. In contrast, C2HS results in no changes in diaphragm muscle fiber cross-sectional areas 2 weeks (Miyata et al., 1995; Zhan et al., 1997) and 4 weeks post-injury (unpublished observations). It has been suggested that the transient hypertrophy of DNV diaphragm fibers is due to passive stretching of the muscle caused by contractions of the intact contralateral hemidiaphragm (Yellin, 1974). However, using sonomicrometry in rabbits, we found that passively lengthening of the midcostal diaphragm was minimal following DNV and resulted in no appreciable strain (Zhan et al., 1995). Moreover, diaphragm fibers in the sternal portion actually shortened passively with continued contraction of the contralateral side. Thus, without mechanical strain, there is no mechanical loading of the paralyzed diaphragm muscle fibers to induce hypertrophy. Furthermore, the fact that hemidiaphragm paralysis induced by C2HS resulted in no transient hypertrophy indicates that mechanical loading is not involved. Based on the similar adaptations elicited by TTX and DNV, it is doubtful that nerve-derived trophic influences are involved in the transient hypertrophy.

Recently, we examined the mechanisms underlying changes in diaphragm muscle protein expression following DNV (Argadine et al., 2009). Overall, protein degradation increased by 5 days after diaphragm DNV, highlighting the importance of protein degradation as a cause of negative protein balance. We previously showed that mRNA expression for the contractile protein myosin heavy chain (MyHC) increased at 1 day after DIAM DNV, but decreased significantly at 3, 7 and 14 days after DNV (Geiger et al., 2003). The correlation between MyHC protein and mRNA levels after DNV was not straightforward. For example, decreased MyHC_{2B} protein expression at 14 days after DNV was associated with unchanged MyHC_{2B} mRNA levels. Thus, post-translational changes that include increased protein degradation are likely involved in the reduced contractile protein expression in the diaphragm after DNV notwithstanding differential transcriptional and translational changes in MyHC gene expression (Argadine et al., 2009). Although similar measurements have not been conducted after TTX, a similar pattern of muscle fiber hypertrophy/atrophy is observed suggesting that the underlying mechanisms are shared. It is possible that with TTX nerve blockade, normal nerve-derived trophic influences are disrupted even though the conduit for axoplasmic flow is not. We recently demonstrated that a nerve-derived trophic influence – neuregulin, does exert a positive effect on protein synthesis in the diaphragm muscle (Hellyer et al., 2006). It is doubtful that other nerve-derived trophic substances such as brain-derived neurotrophic factor (BDNF) or neurotrophin-4 (NT-4) are involved since diaphragm muscle fibers express only the truncated isoforms of the tropomyosin-related kinase receptor type B (TrkB) that lacks the intracellular kinase domain necessary for ligand-induced signaling. Similarly, it is doubtful that NT-3 or nerve growth factor (NGF), other members of the neurotrophin family of trophic factors, are involved in diaphragm muscle adaptations to DNV or TTX nerve blockade since diaphragm expression of their Trk receptors, TrkC and TrkA respectively, is low (unpublished observations). Clearly, the loading or unloading conditions associated with diaphragm muscle paralysis induced by TTX nerve blockade and C2HS may differ from those following DNV. These aspects remain to be elucidated.

4. Neuromuscular junction adaptations to inactivity

Differences in neuromuscular junction morphology and synaptic vesicle pools are present across diaphragm muscle fiber types, which must be considered when examining inactivity-induced synaptic plasticity.

4.1. Morphological adaptations to muscle inactivity

Neuromuscular junctions at type I or IIa fibers display smaller size and fewer branches than those at type IIx and/or IIb fibers (Mantilla et al., 2004a; Prakash et al., 1996; Prakash et al., 1995; Sieck & Prakash, 1997). In general, neuromuscular junctions are ~66% larger at type IIx and/or IIb fibers compared to type I or IIa fibers in control animals. Ultrastructurally, neuromuscular junctions at type I or IIa diaphragm muscle fibers exhibit greater postsynaptic gutter depth and frequent interposition of mitochondria or myonuclei compared to those at type IIx and/or IIb fibers, which generally are larger, displaying direct apposition of motor end-plates and myofibrils (Fahim et al., 1984; Fahim & Robbins, 1982; Mantilla et al., 2004a; Mantilla et al., 2007).

Following diaphragm muscle paralysis induced by either C2HS or TTX nerve blockade there are important differences in neuromuscular junction morphology. Both C2HS and TTX resulted in fiber-type specific changes in neuromuscular junction dimensions, specifically at type IIx and/or IIb fibers (Mantilla et al., 2007; Prakash et al., 1999; Prakash et al., 1995). No differences in axon terminal or motor end-plate size were evident at type I or IIa diaphragm muscle fibers following diaphragm inactivation. In contrast, axon terminals and motor end-plates innervating type IIx and/or IIb fibers increased ~50% following 14 days of C2HS compared to control, with the extent of pre- and post-synaptic overlap also increasing

significantly. Following TTX, axon terminals and motor end-plates at type IIx and/or IIb fibers increased ~35% compared with control and, when normalized to fiber cross-sectional area, the change in neuromuscular junction dimensions was similar to that after C2HS. Taken together, these changes indicate that diaphragm inactivity-induced adaptations in neuromuscular junction morphology depend on fiber type and may reflect preservation of axoplasmic transport of nerve-derived trophic substances (e.g., neurotrophins) in these models.

4.2. Neuroplasticity in synaptic vesicle release

Synaptic vesicle distribution and release at individual neuromuscular junctions also differ across fiber types in the rat diaphragm (Ermilov et al., 2007a; Mantilla et al., 2004a; Rowley et al., 2007). The size of releasable pools of synaptic vesicles at diaphragm neuromuscular junctions differs across fiber types. For example, average FM4-64 uptake is greater in presynaptic terminals at type I or IIa fibers compared to terminals at type IIx and/or IIb fibers (Mantilla et al., 2004a). Based on measurements of active zone distribution and number of “docked” vesicles, we estimated that the total number of vesicles docked at all active zones within a single terminal (i.e. the readily-releasable pool) is ~2x larger at type IIx and/or IIb fibers than at type I or IIa diaphragm fibers (Mantilla et al., 2004a; Rowley et al., 2007). In addition, axon terminals at type IIx and/or IIb fibers display reductions in quantal release during repetitive stimulation that can be accounted for by depletion of docked synaptic vesicles. In contrast, axon terminals at type I or IIa fibers exhibit reductions in quantal release that must also reflect a decrease in the probability of synaptic vesicle release during each electrical stimulus given the larger than expected reduction in quantal release using models of vesicle pool depletion during repetitive stimulation (Rowley et al., 2007). Furthermore, differences in the activation threshold for action potential generation across fiber types result in the safety factor for neuromuscular transmission (i.e. the ratio of end-plate potential amplitude to activation threshold) being larger for type IIx or IIb fibers than for type I or IIa fibers in the rat diaphragm muscle (Ermilov et al., 2007a). Lower action potential thresholds at type IIx and/or IIb fibers than at type I or IIa fibers may reflect greater postsynaptic folding and voltage-gated Na⁺ channels (Ruff, 1992; Wood & Slater, 1995). Thus, the ability to sustain neuromuscular transmission at motor units comprising type I or IIa fibers may relate to slower depletion of releasable synaptic vesicle pools during repetitive activation given their lower firing rates (Mantilla et al., 2004a; Reid et al., 2003).

The size of releasable pools of synaptic vesicles is differentially affected by diaphragm muscle inactivity imposed by C2HS or TTX nerve blockade (Mantilla et al., 2007). At 14 days after C2HS, the pool of releasable synaptic vesicles (measured by uptake of the styryl dye FM4-64) increased significantly at all terminals, but was substantially larger at type IIx and/or IIb fibers (41%) than at type I or IIa diaphragm muscle fibers (17%). Following TTX nerve blockade, the pool of releasable synaptic vesicles decreased significantly at all presynaptic terminals, and there were no longer fiber type differences in releasable pool size. Furthermore, although the size of each active zone did not change following diaphragm muscle paralysis induced by either C2HS or TTX nerve blockade, synaptic vesicle density increased at all pre-synaptic terminals following C2HS (by ~60% at type I or IIa fibers and ~30% at type IIx and/or IIb fibers compared to control) whereas density decreased at all pre-synaptic terminals following TTX (by ~20% at type I or IIa fibers and ~40% at type IIx and/or IIb fibers compared to control). The number of synaptic vesicles directly abutting the active zone (i.e., docked) did not change appreciably following C2HS, but was reduced 20–50% after TTX across all presynaptic terminals independent of fiber type. Overall, these changes in synaptic vesicle pool size correspond with recent electrophysiological measurements obtained at type-identified neuromuscular junctions demonstrating reductions in quantal content after TTX, especially at type IIx and/or IIb fibers (unpublished observations). Thus, it is likely that the size of the different synaptic vesicle pools is regulated by activity at the phrenic motoneuron, and does not depend on diaphragm muscle

activity or muscle-derived trophic substances. In addition, diaphragm muscle paralysis induced by cervical spinal cord injury in the absence of phrenic motoneuron injury result in neuromuscular adaptations that may facilitate functional recovery. Indeed, neuromuscular transmission during repetitive activation of the rat diaphragm muscle is enhanced following C2HS, but is impaired following TTX blockade of the phrenic nerve (Miyata et al., 1995; Prakash et al., 1999).

Diaphragm muscle paralysis induced by C2HS or TTX nerve blockade may also impact the acetylcholine content per vesicle or the size of each vesicle (i.e., quantal size – the amplitude of miniature postsynaptic currents occurring spontaneously thought to correspond to the release of a single synaptic vesicle). Interestingly, acetylcholine release increased after TTX-induced presynaptic inactivity in mice, and this increase did not depend on whether muscle fibers were concurrently inactive or not (Wang et al., 2005). Thus, it is possible that presynaptic activity is important in regulating quantal size. However, in recent electrophysiological studies, we found that miniature end-plate currents were consistently larger at type I or IIa fibers than at type IIx and/or IIb fibers, and, importantly, did not change following C2HS or TTX nerve blockade when compared to control (unpublished observations). Taken together, these results indicate that neuroplasticity in presynaptic terminals at the diaphragm muscle are important in determining neuromuscular function, and must be considered when evaluating different interventions following spinal cord injury, for instance use of neurotrophins to enhance neuromuscular transmission (Ermilov et al., 2007b; Mantilla et al., 2004b) or phrenic nerve pacing.

5. Phrenic motoneuron adaptations to inactivity

Motoneuron morphology varies considerably within a pool, including that of phrenic motoneurons (Burke et al., 1992; Cameron et al., 1983; Cameron et al., 1985; Prakash et al., 2000; Torikai et al., 1996). Differences in soma dimensions and dendritic arborization likely contribute to heterogeneity in intrinsic electrophysiological properties across motoneurons, which may correspond with motor unit type. As the fundamental building blocks of neuromotor control, motor units are generally recruited in orderly fashion (Liddell & Sherrington, 1925), in part reflecting their intrinsic, size-related electrophysiological properties (Henneman et al., 1965). During spontaneous breathing in cats, phrenic motoneurons that are recruited first have slower axonal conduction velocities compared to those motoneurons recruited later (Dick et al., 1987). Similarly, adult rat phrenic motoneurons displaying early discharge within an inspiratory burst (based on their relative onset time) exhibit higher input resistance and lower rheobase than those displaying later inspiratory discharge or none at all (i.e., quiescent motoneurons) (Hayashi & Fukuda, 1995). In general, motoneurons belonging to type S motor units (innervating slow-twitch type I muscle fibers) generally display higher input resistance, lower rheobase and slower axonal conduction velocities compared to motoneurons innervating fast-twitch type II muscle fibers. Among fast twitch motor units, fatigue resistant (FR – innervating type IIa fibers) units have the lowest recruitment threshold, followed in rank order by fatigue intermediate (FInt – innervating type IIx fibers) and highly fatigable (FF – innervating type IIb fibers) units (Burke et al., 1973; Sieck et al., 1989; Sieck et al., 1996). Motor unit heterogeneity is important to accomplish different behavioral tasks (Burke et al., 1973; Sieck & Fournier, 1989). Clearly, normal physiological recruitment of diaphragm motor units provides a distinct advantage to sustain lung ventilation. For instance, activation of highly-fatigable FF motor units in the diaphragm would not permit sustained activation during normal quiet breathing or even during hypoxic conditions. Recruitment of type FF and FInt motor units becomes necessary only during short duration behaviors that require higher force (e.g., coughing) (Sieck & Fournier, 1989). Changes in phrenic motoneuron morphology may thus impact normal recruitment of motor units across both ventilatory and non-ventilatory behaviors.

There is currently no published information on gross morphological changes of phrenic motoneurons in response to inactivation following C2HS. However, there are significant ultrastructural changes in the cervical spinal cord following C2HS (Goshgarian et al., 1989; Hadley et al., 1999). For example, there is a significant increase in the number of “double synapses” and the length of dendro-dendritic appositions over time following C2HS. These changes likely result from interruption of descending excitatory drive, rather than the transection itself, since a cold block induces similar changes (Castro-Moure & Goshgarian, 1997). Retraction of glial processes may facilitate these ultrastructural changes (Goshgarian, 2003; Goshgarian et al., 1989), possibly from activity of extracellular matrix proteases including plasminogen activators (c.f., Seeds et al., 2009). In fact, these ultrastructural changes may unmask previously ineffective, contralateral synaptic connections within the spinal cord restoring some function to the ipsilateral diaphragm, i.e. the so-called “crossed phrenic phenomenon” (c.f., Goshgarian, 2009).

In a recent study using detailed three-dimensional reconstructions of individual phrenic motoneurons, we found that C2HS and TTX nerve blockade result in important morphological differences in soma dimensions and dendritic arborization. After 14 days of diaphragm muscle paralysis, soma and total phrenic motoneuron surface area was moderately, yet significantly, reduced by C2HS compared to sham-operated controls, but increased ~2-fold with TTX nerve blockade (unpublished observations). These results suggest that the morphological adaptations of phrenic motoneurons do not reflect diaphragm muscle inactivity. In addition, these changes likely result in converse changes in motoneuron excitability that would tend to preserve orderly motoneuron recruitment: an overall reduction in motoneuron size after C2HS would increase excitability, favoring early recruitment; an overall increase in motoneuron size after TTX would reduce excitability. These morphological adaptations of phrenic motoneurons to diaphragm muscle inactivity may play an important role in functional recovery following upper cervical spinal cord injury. Interestingly, Saboisky et al. (2007) suggest that, in humans, respiratory motoneuron bursting is determined primarily by an inhomogeneous distribution of descending premotor inputs rather than intrinsic, size-related properties of phrenic motoneurons. Single motor unit recordings derived from the raw EMG signal were obtained only when they were uniquely identifiable, and thus have significant limitations, including sampling bias in favor of units with the highest threshold and lack of information on frequency coding of motor unit recruitment. Regardless, diaphragm EMG amplitude increases across behavioral conditions from eupnea to hypoxia-hypercarbia to airway occlusion in control animals, consistent with the increasing force generated by the diaphragm in these conditions (Dow et al., 2006; Sieck & Fournier, 1989). Using EMG recordings in rats displaying functional recovery of rhythmic diaphragm activity after C2HS, we recently found that diaphragm EMG activity was of reduced amplitude compared to control, and, although blunted, EMG activity increased similarly across the different behavioral conditions (unpublished observations). These findings suggest that functional recovery results from a generalized strengthening of descending presynaptic drive to phrenic motoneurons rather than an inhomogeneous distribution of synaptic inputs across motor units, and is thus consistent with functional recovery post-injury reflecting the overall increase in motoneuron excitability that is expected from a reduction in phrenic motoneuron dimensions.

6. Mechanisms underlying phrenic motor unit plasticity

Motor unit adaptations to altered activity may be mediated via several mechanisms (see (Mantilla & Sieck, 2003). In this regard, the role of both nerve-derived and muscle-derived trophic factors has been the focus of intense investigation (c.f., Mantilla & Sieck, 2008a). Importantly, trophic factors may exert local as well as distant effects. Several studies reported changes in the expression of trophic factors including BDNF, NT-3 and NT-4 (Satake et al., 2000; Widenfalk et al., 2001). Most of these studies, however, reported limited temporal

expression profiles for mRNA rather than protein levels. Discrepancies in cellular substrates displaying altered trophic factor expression as well as in the direction of these changes are consistent with a complex process that differs across experimental models (e.g., cervical vs. thoracic transection, mechanical vs. traumatic injury), species, and time post-injury.

In several recent studies, Mitchell and colleagues examined the role of neurotrophins in the neuroplasticity of phrenic motor output. For example, new BDNF synthesis in the spinal cord was necessary for long-term facilitation of phrenic motor output induced by intermittent hypoxia (Baker-Herman et al., 2004). In addition, BDNF application to the cervical spinal cord was sufficient to elicit an enhancement of phrenic motor output suggestive of long-term facilitation. More recently, facilitation of phrenic motor output was also induced after transactivation of TrkB receptors by adenosine 2a receptor ligands (Golder et al., 2008). However, the exact cellular substrate(s) for these forms of phrenic motoneuron plasticity are still unclear. In addition, it seems important to determine the expression of neurotrophins and their receptors following C2HS or TTX as it is possible that following C2HS increased expression of NT-4, BDNF and/or TrkB (and, conversely, decreased expression following TTX) may contribute to the morphological and functional plasticity at phrenic motoneurons and diaphragm neuromuscular junctions. Novel techniques such as laser capture microdissection permit selective examination of retrogradely-labeled phrenic motoneurons for mRNA measurements. In addition, experimental changes in neurotrophin signaling are possible using intrapleural-based retrograde targeting of phrenic motoneurons with siRNA or viral constructs (Mantilla et al., 2009).

Importantly, several studies show that delivery of combinations of trophic factors increases motoneuron survival and axon sprouting within the injured cord (Bregman et al., 1997; Iarikov et al., 2007; Lu et al., 2003; Novikova et al., 2002; Ruitenberg et al., 2004). Unfortunately, functional recovery does not represent only increased motoneuron survival or even axonal sprouting since physiologic integration of the complex circuitry involved in motor control may be necessary. Detailed studies reporting motoneuron expression of trophic factors or their receptors following spinal cord injury are necessary to understand the mechanisms underlying motoneuron-specific adaptations to inactivity, especially at phrenic motoneurons (c.f., Sieck and Mantilla, 2009). In this sense, it is highly likely that therapeutic strategies to restore rhythmic diaphragm activity following spinal cord injury should vary depending on interval time post-injury. For example, we found that mRNA expression of the full-length isoform of the TrkB receptor increased in microdissected phrenic motoneurons at 3 days post-C2HS. In addition, TrkB phosphorylation increased in the cervical spinal cord ventral horn region containing phrenic motoneurons. These results are consistent with a progressive enhancement of TrkB signaling post-C2HS, which we believe accounts for the time course of functional recovery of rhythmic diaphragm activity after injury.

An increase in neurotrophin signaling post-C2HS may itself promote TrkB receptor expression in phrenic motoneurons – a positive feedback effect. For example, *in vitro* studies using PC12 and cultured neocortical cells demonstrated that neurotrophins can induce further expression of neurotrophins, as well as an upregulation of their receptors (Canossa et al., 1997; Rankin et al., 2008). To the best of our knowledge, no studies have examined neurotrophin-induced receptor upregulation *in vivo*. Motoneuron expression of trophic factors may also be important for neighboring cells. For instance, motoneuron-derived NT-3 can serve as a survival factor for spinal interneurons (Bechade et al., 2002). Clearly, complex trophic interactions are at play within the spinal cord in the environment surrounding phrenic motoneurons following spinal cord injury even when the level injury is cephalad to the region containing the motoneuron pool (e.g., C2HS). The role of inactivity in altered trophic factor or receptor expression by motoneurons has also not been ascertained. Neurotrophin synthesis and release are regulated by neuronal activity in the hippocampus (Kang & Schuman, 1995), but whether this also occurs

at motoneurons is not clear. Local availability of trophic factors including those derived from motoneurons themselves can affect presynaptic terminals, interneurons and surrounding glial cells, and these trophic factor effects can both positively and negatively alter functional recovery.

Neurotrophins can directly modulate synaptic efficacy. For example, BDNF stimulates synapsin I phosphorylation in a mitogen activated protein kinase-dependent manner (Jovanovic et al., 2000), regulating neurotransmitter release. In adult rat diaphragm-phrenic nerve preparations, both BDNF and NT-4 improved neuromuscular transmission in a TrkB-dependent manner (Ermilov et al., 2007b; Mantilla et al., 2004b). This effect of BDNF is presumably presynaptic, but whether neurotrophins also regulate the size of releasable synaptic vesicle pools, and thus contribute to the differences observed across the C2HS and TTX models of diaphragm muscle inactivity, has not been determined directly. In culture, neuromuscular junctions formed onto amphibian myocytes overexpressing NT-4 show both higher levels of spontaneous synaptic activity and enhanced evoked synaptic transmission when compared to synapses formed onto neighboring myocytes not overexpressing NT-4 (Wang & Poo, 1997). In the both the C2HS and TTX models, the potential for nerve-derived trophic influences remained intact since axoplasmic transport was unaffected (Mantilla et al., 2007; Miyata et al., 1995; Prakash et al., 1999; Zhan & Sieck, 1992). However, trophic factor synthesis and release may depend on motoneuron activity, and these possibilities merit direct examination. In addition, whether there are motor unit type differences in trophic factor expression are not clear (Mantilla & Sieck, 2008a).

Neurotrophins might also indirectly influence diaphragm fiber size. For instance, of BDNF/TrkB signaling may enhance release of other nerve-derived trophic factors (e.g., NRG). This indirect effect has never been explored. Alternatively, it is possible that BDNF/TrkB signaling affects diaphragm fiber activity levels. However, we have shown that unilateral diaphragm inactivity induced by C2HS does not result in any significant muscle fiber atrophy on the affected side (Zhan et al., 1997). Furthermore, diaphragm inactivity induced by C2HS, TTX or DNV result in increased contralateral diaphragm activity, usually by at least 50% compared to control (Miyata et al., 1995; Prakash et al., 1999), and this increase in activity is not associated with any significant change in muscle fiber size (Zhan et al., 1997). Whether NRG is released from motoneurons depending on motoneuron activity or motor unit type is not clear. During embryonic development, motoneuron expression of NRG variants important for neuromuscular junction formation peaks at the time of first muscle contact, and, in culture, BDNF and NT-3 increase NRG expression by motoneurons (Loeb & Fischbach, 1997). Muscle fiber inactivity induced by blocking muscle acetylcholine receptors reduced muscle expression of BDNF and NT-3, and reduced NRG expression at neuromuscular junctions (Loeb et al., 2002). The interaction between neurotrophin-mediated and NRG-dependent effects on motor unit plasticity clearly deserves further study.

The interaction between trophic influences, rehabilitation strategies and functional electrical stimulation for functional recovery following spinal cord injury has recently received considerable attention (c.f., Edgerton et al., 2008). However, specific studies of trophic factor infusion in the context of either phrenic nerve pacing or epidural stimulation are still lacking. Important recent developments are very promising. For instance, using a novel method of epidural stimulation, asynchronous activation of phrenic motor neurons presumably following a physiological recruitment order was recently demonstrated (DiMarco & Kowalski, 2009). A major advantage of this method is that diaphragm muscle activity can be restored providing adequate ventilation without eliciting fatigue for a much longer period. Importantly, preservation of functional reserve capacity may allow expulsive motor behaviors such as cough (Sieck & Mantilla, 2009a), and hopefully reduce morbidity and mortality following spinal cord injury.

7. Conclusions

The diaphragm muscle is a highly active muscle, making diaphragm motor units particularly responsive to inactivity and/or trophic influences. Importantly, inactivity-induced neuromuscular plasticity reflects specific adaptations that depend on inactivity at the motoneuron rather than at axon terminals or muscle fibers. It may be possible to maintain neuromuscular transmission and functional properties of diaphragm muscle fibers after spinal cord injury when motoneurons are not injured directly, thus, providing a substrate for functional recovery and/or specific therapeutic approaches such as phrenic nerve pacing. For instance, the relative preservation of neuromuscular junctions at type I and IIa fibers suggests a potential for functional recovery following prolonged respiratory muscle paralysis, and the enhanced ability to sustain neuromuscular transmission during repetitive activation may represent another strategy to enhance functional recovery following spinal cord injury. In particular, these findings suggest that interventions that capitalize on the physiological recruitment of motor units (e.g. via epidural stimulation rather than phrenic nerve pacing, see DiMarco & Kowalski, 2009) deserve further examination in conjunction with strategies targeting increased expression of trophic substances.

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Figure 1.

Schematic depicting different experimental models of diaphragm muscle (DIAM) inactivity. Following a cervical spinal cord hemisection at C2 (C2HS; left), descending excitatory input to phrenic motoneurons (PhrMn) is interrupted and all components of DIAM motor units (PhrMn, neuromuscular junction – NMJ and DIAM fibers) become inactive. With tetrodotoxin-induced conduction blockade (TTX; right) of the phrenic nerve, DIAM fibers and NMJs become inactive, but PhrMn remain active generating action potentials in response to descending excitatory input. Neuromuscular adaptations to inactivity of the DIAM are summarized below (Mantilla et al., 2007; Miyata et al., 1995; Prakash et al., 1999; Zhan & Sieck, 1992). Although not shown, morphological adaptations of DIAM fibers to phrenic denervation (DNV; not shown) are consistently similar to TTX.

Figure 2.

Confocal photomicrographs of adult rat motor end-plates (labeled with α -bungarotoxin, red) and axon terminals (labeled with FM4-64, green) at diaphragm muscle fibers from control, C2HS and TTX rats (Mantilla et al., 2007). Notice the differences in size and complexity (i.e. branching) evident across axon terminals at type I or IIa fibers (top left in each panel) vs. type IIx and/or IIb fibers (bottom right in each panel). Axon terminals are shown in green and the fluorescence intensity correlates with the size of the releasable synaptic vesicle pool. Notice the increase in the size of the releasable synaptic vesicle pool after C2HS compared to control, and the reduction in synaptic vesicle pool size following TTX nerve blockade. All photomicrographs are maximum projection images of confocal stacks. Scale bar for all panels: 15 μ m.