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Trophic Factor Expression in Phrenic Motor Neurons

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Abstract

The function of a motor neuron and the muscle fibers it innervates (i.e., a motor unit) determines neuromotor output. Unlike other skeletal muscles, respiratory muscles (e.g., the diaphragm, DIAM) must function from birth onwards in sustaining ventilation. DIAM motor units are capable of both ventilatory and non-ventilatory behaviors, including expulsive behaviors important for airway clearance. There is significant diversity in motor unit properties across different types of motor units in the DIAM. The mechanisms underlying the development and maintenance of motor unit diversity in respiratory muscles (including the DIAM) are not well understood. Recent studies suggest that trophic factor influences contribute to this diversity. Remarkably little is known about the expression of trophic factors and their receptors in phrenic motor neurons. This review will focus on the contribution of trophic factors to the establishment and maintenance of motor unit diversity in the DIAM, during development and in response to injury or disease.

Keywords

Respiratory muscles; diaphragm; skeletal muscle; development; neurotrophin; neuregulin; glial cell line-derived neurotrophic factor

1. Introduction

The function of a motor neuron and the muscle fibers it innervates (i.e., a motor unit) are important determinants of respiratory neuromotor output. Respiratory muscles, unlike other skeletal muscles, must function from birth onwards and commonly display high levels of activity. Indeed, as the major inspiratory muscle in mammals, the diaphragm muscle (DIAM) is uniquely active and rat DIAM motor units display a daily duty cycle (ratio of active to inactive time) ~ 35% (Kong et al., 1986). In contrast, rat hind limb muscles display duty cycles ranging from ~2% for the extensor digitorum longus (EDL) muscle to ~14% for the soleus muscle (Hensbergen et al., 1997). DIAM motor units are involved in a number of ventilatory and non-ventilatory behaviors, including vocalization, swallowing, and expulsive behaviors involved in airway clearance (Sieck et al., 1989a). At present, the mechanisms underlying the development and regulation of motor unit diversity (necessary to provide for this broad range of motor behaviors) are not well understood. Trophic factor influences may contribute to this diversity and this review will focus on the expression of trophic factors and their effects on phrenic motor neurons during development and in response to injury or disease.

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Phrenic motor neurons innervating the DIAM are located within lamina IX of cervical spinal cord segments C₃-C₅ in rats (Prakash et al., 2000; Song et al., 2000), C₄-C₆ in cats (Webber et al., 1979), C₅-C₇ in ferrets (Yates et al., 1999), and C₃-C₅ in humans (Keswani et al., 1955). Phrenic motor neurons receive rhythmic excitatory drive from premotor neurons located in the medulla (Feldman et al., 1985; Ellenberger et al., 1988). Thus, trophic factors influencing phrenic motor neurons might derive from peripheral, segmental or central sources (Fig. 1). *Peripheral* sources include DIAM fibers, Schwann cells in the phrenic nerve and circulating (systemic) factors. *Segmental* sources include surrounding glia, interneurons and afferent neurons with their cell bodies in neighboring segments of the spinal cord and in dorsal root ganglia. *Central* sources derive predominantly from supraspinal sources such as the rostral ventral respiratory group in rats (Ellenberger et al., 1990; Dobbins et al., 1994) or the ventral and dorsal respiratory groups and medial reticular formation in cats and ferrets (Feldman et al., 1985; Yates et al., 1999). Any and all of these sources may provide trophic support to or derive trophic support from phrenic motor neurons.

Members of a number of trophic factor families are now known to exert trophic influences on motor neurons (Oppenheim, 1996). What is less clear is the cellular substrate for trophic effects of these factors, either acting directly on motor neurons or indirectly via effects on neighboring cells in the environment surrounding motor neurons. In this review, we will explore the contribution of trophic factors to the establishment and maintenance of motor unit diversity.

2. Classification of DIAM motor units

Neural control of the DIAM is based on recruitment and frequency coding of motor units, which in the adult DIAM display considerable diversity in their mechanical, histochemical and biochemical properties (Fournier et al., 1988; Sieck, 1991; Sieck, 1994; Su et al., 1997; Butler et al., 1999). This heterogeneity controls the range of muscle force generation required during different motor behaviors. Indeed, the combined contractile and fatigue properties of a motor unit pool determine the limits under which a muscle can respond to the varying mechanical demands placed upon it. Clearly these motor demands change during development and DIAM motor units must adapt or remodel to accommodate these changing demands.

Motor units are commonly classified into different types according to the mechanical and fatigue properties of their muscle fibers (Burke, 1981; Fournier et al., 1988; Sieck et al., 1989b): 1) slow-twitch, fatigue resistant (type S), 2) fast-twitch, fatigue resistant (type FR), 3) fast-twitch, fatigue-intermediate (type FInt), and 4) fast-twitch, fatigable (type FF). This diversity is manifest in structural and functional differences across muscle fibers, classified histochemically or according to myosin heavy chain (MHC) isoform expression (Johnson et al., 1994; Sieck et al., 1996; Geiger et al., 2000). The innervation ratio (i.e., the number of muscle fibers innervated by a motor neuron) varies across muscles, ranging from 10 or less in hand and eye muscles to hundreds in limb and postural muscles (Sieck, 1988). Within a muscle, innervation ratio is greater at type FInt and FF motor units compared to type S and FR units. In muscles of mixed composition, differences in fiber size also exist across motor units with fibers of type FInt and FF units (expressing MHC_{2X} and MHC_{2B} isoforms) displaying greater cross-sectional area than fibers of type S and FR units (expressing MHC_{Slow} and MHC_{2A} isoforms, respectively). Specific force (i.e., force per unit cross-sectional area) varies across fibers, with that of fibers expressing MHC_{2X} and/or MHC_{2B} being greater than that of fibers expressing MHC_{Slow} or MHC_{2A} (Geiger et al., 2000). Together, the greater innervation ratio, larger fiber size and greater specific force contribute to greater forces being generated by type FInt and FF motor units compared to type S and FR units.

Motor unit composition is fundamental in determining the functional capacity of the DIAM in accomplishing different motor behaviors. The overall force generated by a muscle results from

the recruitment of motor units in an orderly fashion (Sieck et al., 1989a; Butler et al., 1999). Consistent with the “size principle” (Henneman et al., 1965; Gordon et al., 2004), recruitment of motor units generally matches their mechanical and fatigue properties: type S and FR motor units are recruited first, followed by type FIInt and FF units. Forces generated during most sustained motor behaviors in the DIAM only require recruitment of type S and FR motor units. Only during high force, short duration motor behaviors does recruitment of more fatigable type FIInt and FF units become necessary (Sieck et al., 1989a). The mechanisms underlying the development and maintenance of motor unit diversity are not well understood. Trophic influences may play an important role in defining motor unit diversity.

3. Trophic factors and their receptors

In very general terms, trophic factors stimulate proliferation, promote cell survival and differentiation, and regulate cell function. Many trophic factors were first identified by their roles in sustaining survival of specific neuronal populations during development (Hamburger, 1992). However, trophic factors are now known to exert additional effects including regulation of synaptic efficacy in the adult. The abundance of trophic factors in the nervous system suggests that they exhibit both unique roles as well as overlapping effects on their target cells, for instance motor neurons (Oppenheim, 1996; Baloh et al., 2000; Sendtner et al., 2000; Buonanno et al., 2001).

The sites of action of trophic factors have not been unambiguously ascertained. For instance, trophic factors could be released by motor neuron dendrites or cell bodies and act retrogradely on interneurons, descending afferent axons or surrounding glia (Yan et al., 1993; Rind et al., 2005). Alternatively, trophic factors could be transported anterogradely in motor axons and affect distal targets, including skeletal muscle and perisynaptic Schwann cells (Russell et al., 2000; Mantilla et al., 2004b; Hellyer et al., 2006). Several trophic factors and/or their receptors display widespread expression across motor units and will be discussed briefly (Fig. 1).

3.1. Neurotrophin family

The family of neurotrophins includes nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and NT-4 (Reichardt, 2006). Neurotrophins exert their effects through a low affinity receptor (p75) or a family of high affinity tropomyosin-related kinase (Trk) receptors. Trk receptors exhibit the following selectivity: TrkA preferentially binds NGF, TrkB binds BDNF and NT-4, and TrkC binds NT-3. Neurotrophins are synthesized as immature pro-neurotrophins, which are cleaved by furin and other proteases to their mature form (Lee et al., 2001b). Pro-neurotrophins such as pro-NGF and pro-BDNF induce apoptosis by activating a p75 receptor-sortilin complex (Lee et al., 2001b; Teng et al., 2005), but their physiological roles are poorly understood (Jansen et al., 2007).

The expression of neurotrophins and Trk receptors has been extensively studied, particularly during development (Funakoshi et al., 1993; Timmusk et al., 1993; Griesbeck et al., 1995; Erickson et al., 1996; Ip et al., 2001). Neurotrophins are produced by motor neurons (Henderson et al., 1993; Koliatsos et al., 1993). In fact, BDNF and NT-3 immunoreactivity is present in large neurons (possibly phrenic motor neurons) in the cervical ventral spinal cord (Johnson et al., 2000). The neurotrophins BDNF, NT-3 and NT-4 are also expressed in skeletal muscle (Henderson et al., 1993; Griesbeck et al., 1995), but it is unclear whether there are differences in neurotrophin expression across motor unit types (Funakoshi et al., 1995; Sakuma et al., 2001; Vernon et al., 2004).

Alternative splicing of Trk receptor mRNA yields both full-length (catalytically active) and truncated forms lacking the intracellular tyrosine kinase domain (Barbacid, 1994). Neurotrophin binding to Trk receptors is thought to result in receptor dimerization and

transactivation at the cell surface, with subsequent internalization (Reichardt, 2006). Recently, TrkB receptors have also been implicated in neurotrophin-independent signaling via interactions with Gs-coupled receptors and transactivation of intracellular TrkB (Lee et al., 2001a; Rajagopal et al., 2004; Golder et al., 2008).

The receptor TrkB is present at both pre- and postsynaptic sites of neuromuscular junctions at both slow and fast muscle fibers, as well as in perisynaptic Schwann cells (Frisen et al., 1993; Funakoshi et al., 1993; Escandon et al., 1994; Gonzalez et al., 1999). It is unclear whether there are differences in expression of the full-length vs. truncated forms of neurotrophin receptors in these different components of the neuromuscular junction (Funakoshi et al., 1995; Gonzalez et al., 1999; Sakuma et al., 2001).

3.2. Transforming growth factor family (TGF β)

The TGF β family of trophic factors includes glial cell line-derived neurotrophic factor (GDNF), neurturin, artemin and persephin (Baloh et al., 2000; Takahashi, 2001; Sariola et al., 2003). Members of the TGF β family bind GDNF family receptors (GFR), which signal by dimerizing with the rearranged in transfection (RET) receptor tyrosine kinase. There are four glycosyl-phosphatidylinositol-linked GFR receptors (GFR α 1-4), which are membrane-anchored and bind their ligands with different affinities: GDNF preferentially binds to GFR α 1, neurturin to GFR α 2, artemin to GFR α 3 and persephin to GFR α 4 (Baloh et al., 2000; Takahashi, 2001). However, GDNF can also bind GFR α 2, and neurturin can bind GFR α 1, albeit with lower affinities (Sariola et al., 2003). The receptors GFR α 1 and RET show widespread expression in cranial and spinal motor neurons, but only a small subset express GFR α 2 (Golden et al., 1999; Oppenheim et al., 2000). GDNF and neurturin are expressed in skeletal muscle and Schwann cells both during development and in the adult (Henderson et al., 1994; Nguyen et al., 1998; Suzuki et al., 1998). GDNF produced by Schwann cells can be transported retrogradely and anterogradely within motor neurons (Russell et al., 2000). Given that the RET receptor is not expressed in peripheral targets such as Schwann cells or muscle fibers in the tongue or DIAm (Russell et al., 2000), it is not clear whether peripherally delivered GDNF acts only axon terminals themselves (Nguyen et al., 1998; Yang et al., 2004). Recent studies indicate that GFR α 1 might act independent of RET by complexing with neural cell-adhesion molecule NCAM (Paratcha et al., 2003; Sariola et al., 2003), but whether this effect occurs postsynaptically in muscle fibers has not been explored.

3.3. Epidermal growth factor (EGF) family

Neuregulins (NRG) belong to the larger EGF family of trophic factors and activate receptor tyrosine kinases of the ErbB family (Zhu et al., 1995; Trinidad et al., 2000). Four NRG genes exist (NRG1-4) with alternative splicing resulting in multiple NRG isoforms, all of which share EGF-like binding domains (Falls et al., 1993; Buonanno et al., 2001). There are four members in the ErbB receptor family: epidermal growth factor (EGF) receptor (EGFR, also referred to as ErbB1), ErbB2/Neu, ErbB3 and ErbB4 (Yarden et al., 2001). A number of ligands bind to ErbB receptors including all NRG isoforms but with varying affinities and differential signaling effects (Jones et al., 1999). The ErbB1 receptor does not appreciably bind either NRG-1 or NRG-2, consistent with the very limited effects of its prototypical ligand (EGF) in the central nervous system (Falls, 2003). Both NRG-1 and NRG-2 bind to the extracellular domain of ErbB3 or ErbB4 receptors leading to dimerization preferentially with ErbB2 (Graus-Porta et al., 1997; Olayioye et al., 1998). Although the ErbB2 receptor shows no ligand binding affinity itself, upon heterodimerization it greatly increases NRG binding affinity of the ErbB receptor complex (Jones et al., 1999). For example, ErbB2/ErbB3 or ErbB2/ErbB4 heterodimers display ~100-fold higher affinity for NRG-1 β than ErbB3 or ErbB4 homodimers (Sliwkowski et al., 1994; Jones et al., 1999). Similarly, NRG-2 binds ErbB2/ErbB4 heterodimers with greatest affinity (Tzahar et al., 1996; Jones et al., 1999). Consequently, cells co-expressing ErbB2 with

ErbB3 or ErbB4 would preferentially form ErbB2/ErbB3 or ErbB2/ErbB4 heterodimers after being exposed to NRG-1, and ErbB2/ErbB4 heterodimers following NRG-2. Importantly, distinct signaling pathways are activated depending on the ErbB receptor complex formed by NRG binding (Li et al., 2007).

Previous studies have shown that NRG-1 (both α and β isoforms) and NRG-2 β are expressed by spinal motor neurons (Buonanno et al., 2001; Falls, 2003; Rimer et al., 2004). Schwann cells also express both NRG-1 and NRG-2, whereas muscle fibers reportedly express NRG-1 (Falls et al., 1993). ErbB2, ErbB3 and ErbB4 are expressed in spinal motor neurons (Ricart et al., 2006) and mature skeletal muscle (Moscoso et al., 1995; Zhu et al., 1995; Lebrasseur et al., 2003), including rat DIAM (Hellyer et al., 2006). The functional implications of the different NRG isoforms are incompletely understood, at least in part because of the widespread and seemingly redundant expression of NRG isoforms and ErbB receptors.

3.4. Other trophic factors

Members of several different families of trophic factors and cytokines have received considerable attention primarily as survival factors for motor neurons (Oppenheim, 1996; Sendtner et al., 2000; Brunet et al., 2007). In this regard, individual trophic factors likely exhibit both unique and overlapping effects on survival that are difficult to ascertain unequivocally. Indeed, a number of receptor complexes have been shown to interact with one or more trophic factors to elicit beneficial survival effects. Trophic factors such as vascular endothelial growth factor (Storkebaum et al., 2005), basic fibroblast growth factor (Teng et al., 1999), insulin-like growth factors (Neff et al., 1993), cardiotrophin-1 (Oppenheim et al., 2001), ciliary-neurotrophic factor - CNTF (Oppenheim et al., 1991) and leukemia inhibitory factor (Li et al., 1995) are known to exert significant survival effects both in culture and in vivo. In fact, trophic factors have been used in the treatment of human motor neuron diseases such as amyotrophic lateral sclerosis (Miller et al., 1996; Borasio et al., 1998; Ochs et al., 2000). Unfortunately, these studies had limited success. Although of interest, discussion of trophic factors in the context of motor neuron degeneration is outside the scope of this review. At present, there is insufficient information to determine whether any of these trophic factors may play a role in defining motor unit properties. Thus, these other trophic factors will not be discussed further.

4. Role of trophic factors during development

Although a number of growth factors are known to be involved in motor neuron survival and formation of the neuromuscular junction (Oppenheim, 1996; Sariola et al., 2003), far less is known about the role of trophic factors in the growth of motor neurons or muscle fibers, or in their specialization into motor unit types. For instance, trophic factors may contribute to motor unit type differences via selective expression of specific factors, their receptors or individual signaling pathway components. These are important aspects that are poorly understood.

Phrenic motor neurons exhibit significant growth during postnatal development (Cameron et al., 1990; Cameron et al., 1991; Prakash et al., 2000). For example, rat phrenic motor neurons increase in both soma and dendrite dimensions between postnatal day 21 and adulthood, with significant and proportionate growth of muscle fibers (Prakash et al., 2000). This proportionate growth indicates that even during postnatal development, it is important to match motor neuron and muscle fiber size. In addition, there is greater heterogeneity in phrenic motor neuron size in the adult, with the variance in the distribution of total surface area increasing ~4-fold (Prakash et al., 2000). The characteristic heterogeneity of motor unit types develops following weaning in the rat DIAM when motor unit innervation ratio is fixed (Sieck et al., 1991; Prakash et al., 1993; Mantilla et al., 2008). Before weaning, it is difficult to determine whether synapse elimination and/or secondary myogenesis contribute to changes in motor unit size.

Synapse elimination may affect motor neuron size via an effect on innervation ratio, assuming that trophic support depends on the number of muscle fibers innervated by a motor neuron. For instance, increased innervation ratio resulting from partial denervation of hindlimb muscles and concomitant sprouting of spared axons is associated with increases in motor neuron size (Tissenbaum et al., 1991). In rat DIAM, synapse elimination continues through postnatal day 14; thus, if anything, innervation ratio is reduced during the postnatal period, and this would tend to decrease phrenic motor neuron size - just opposite to what is observed (Cameron et al., 1990; Cameron et al., 1991; Prakash et al., 2000). Postnatal phrenic motor neuron growth may thus depend on specific trophic influences not solely related to innervation ratio. Similarly, it seems reasonable to assume that most trophic effects derive from sources providing the greatest number of synaptic inputs to motor neurons, but it is possible that trophic influence does not correspond with the sheer number of synaptic connections onto motor neurons. This issue deserves further study.

Whether differences in descending excitatory input, activation of specific muscle groups or other activity-related effects play a role in the specialization of motor unit types is not presently known. Early in development, phrenic motor neurons are activated by rhythmic motor patterns generated in the spinal cord (Greer et al., 1992). Subsequently, fetal respiratory movements appear and they increase in frequency and regularity during the prenatal period. It is possible that the growth and maturation of motor neurons depends on these rhythmic motor patterns, either directly via activity-dependent effects or indirectly via neurotransmitter- or trophic factor-mediated effects. During postnatal development, DIAM motor unit activity changes as functional demands shift to include a wider range of ventilatory and non-ventilatory behaviors (Greer et al., 1992; Greer et al., 2006; Mantilla et al., 2008). Accordingly, activation of nearly all DIAM motor units is necessary in neonates during resting ventilation in order to overcome their reduced lung compliance and chest wall stiffness. The more homogeneous and smaller size of phrenic motor neurons in the early postnatal period is consistent with increased excitability (Henneman et al., 1965; Su et al., 1997), and can facilitate synchronous recruitment of DIAM motor units. As neonates mature, their lung compliance and chest wall stiffness increase, and more selective activation of DIAM motor units likely occurs during resting ventilation. Indeed, in the adult rat DIAM only 25-30% of motor units are activated during eupneic breathing (Sieck et al., 1989a; Sieck et al., 1996). Recruitment of additional motor units is necessary only when engaging in more forceful motor behaviors (e.g., coughing, sneezing). Clearly, trophic factor influences may act in an activity-dependent fashion, further complicating unambiguous interpretation of any developmental effects on motor units. Studies using targeted deletions of specific trophic factors or their receptors in the absence of changes in activity levels might help elucidate these issues. Several important questions remain to be answered, including: What are critical trophic influences on motor neurons? Are there developmental windows for a specific form of trophic support? Do trophic factors present in motor neurons exert an effect directly on motor neurons or only indirectly via an effect on the environment surrounding motor neurons?

4.1. Trophic factor effects on motor neuron elimination

The importance of target-derived factors for motor neuron survival was initially revealed by axotomy and limb bud ablation experiments demonstrating increased motor neuron loss during the so-called postnatal period of programmed motor neuron cell death (Hamburger, 1992; Oppenheim, 1996; Sendtner et al., 2000). However, it is not clear whether all motor neuron pools, specifically phrenic motor neurons, are similarly affected by deprivation of target-derived trophic support (Oppenheim, 1986). Regardless, a number of trophic factor families have demonstrated survival-promoting effects both in avian and mammalian model systems (Oppenheim, 1996; Brunet et al., 2007). The survival effects of individual trophic factors on motor neurons during this period of naturally occurring cell death are difficult to study

explicitly given the abundance of trophic factors and their likely overlapping effects (Oppenheim, 1996; Sendtner et al., 2000; Brunet et al., 2007).

Several lines of evidence suggest that neurotrophins are dispensable for maintenance of motor neuron numbers during the postnatal period of motor neuron elimination, despite demonstrated survival-promoting effects of neurotrophins like BDNF (Oppenheim et al., 1992; Brunet et al., 2007) or the widespread distribution of neurotrophins and Trk receptors (Funakoshi et al., 1993; Timmusk et al., 1993; Griesbeck et al., 1995; Erickson et al., 1996; Ip et al., 2001). During postnatal development, mRNA expression for BDNF and NT-3 gradually decreases in both the rat soleus and gastrocnemius muscles, although NT-4 expression increases (Funakoshi et al., 1995). Importantly, mice genetically deficient in NT4 have normal numbers of spinal motor neurons (Conover et al., 1995). Although there is motor neuron loss in knockout mice lacking BDNF, it occurs only during periods of naturally occurring cell death, and likely results from reduced trophic support from peripheral tissues rather than a direct effect on motor neuron survival (Ernfors et al., 1994; Erickson et al., 1996).

In contrast, GDNF seems necessary for survival of certain populations of spinal motor neurons (Garces et al., 2000; Oppenheim et al., 2000; Takahashi, 2001). Indeed, GDNF is one of the most potent survival factors for cultured embryonic spinal motor neurons (Henderson et al., 1994; Brunet et al., 2007). For example, whereas RET is expressed by nearly all motor neurons early in embryological development, GFR α 1 and GFR α 2 display distinct expression patterns with motor neurons expressing one or both receptors (Oppenheim et al., 2000; Homma et al., 2003). Most GDNF-dependent motor neurons require GFR α 1 for their survival response to GDNF or neurturin (Garces et al., 2000). Loss of neurturin or GFR α 2 does not seemingly result in altered trophic response or motor neuron loss (Heuckeroth et al., 1999; Rossi et al., 1999). In a recent study, motor neuron loss in GDNF-, RET- and GFR α 1-deficient mice was restricted to muscle spindle-innervating motor neurons (Gould et al., 2008). Whether a subset of phrenic motor neurons is specifically dependent on GDNF is presently unclear.

NRG-1 effects on acetylcholine receptor (AChR) clustering in muscle fibers have been extensively studied (Sandrock et al., 1997; Sanes et al., 2001; Falls, 2003), however much less is known about other possible effects of NRG-1 on motor neurons. Recently, NRG-1 β was shown to inhibit apoptosis of cultured rat motor neurons in a phosphatidylinositol-3-kinase (PI3K)-dependent manner (Ricart et al., 2006). Importantly, NRG-1 β acted synergistically with GDNF to promote motor neuron survival. In contrast, the neurotrophins BDNF and NGF, acting via the p75 receptor, were pro-apoptotic and completely abolished NRG-1 pro-survival effects by activating the c-Jun N-terminal kinase mitogen-activated protein kinase (MAPK) pathway with induction of neuronal nitric oxide synthase and peroxynitrite formation. Although not specifically examined in phrenic motor neurons, spinal motor neurons in rats and mice express both neurotrophin and ErbB receptors (Rimer et al., 1998; Copray et al., 2000; Pearson et al., 2004), thus providing the substrate for such an effect in vivo. Whether a similar interaction between different trophic factor pathways exists during periods of motor neuron loss (e.g., during postnatal development) is yet to be determined. The interaction between these and other trophic factor families on motor neuron survival is still poorly understood. Trophic factor effects may depend on the specific model used in the assessment of motor neuron survival (Brunet et al., 2007).

4.2. Trophic factors and motor unit specialization

Beyond their possible effects in regulating motor neuron survival during postnatal development, trophic factors may also contribute to the coordinated postnatal growth of motor neurons and muscle fibers (Mantilla et al., 2008). Several studies have examined differences in expression of trophic factors and their receptors in the neonate and in the adult (Golden et al., 1999; Buck et al., 2000; Ip et al., 2001; Hess et al., 2007). Whether differences exist in

neurotrophin expression across motor neurons innervating different motor unit types is controversial. Postnatal expression of NT-4 mRNA increased in hindlimb muscles (Funakoshi et al., 1995), with higher expression in the soleus (predominantly expressing MHC_{Slow}) than in the gastrocnemius (predominantly expressing MHC_{2X} and/or MHC_{2B}). In agreement, NT-4 immunoreactivity was limited to muscle fibers histochemically classified as belonging to type S motor units (Funakoshi et al., 1995). However, when motor neuron size was used to differentiate mRNA expression of neurotrophin and Trk receptors in adult hindlimb motor neurons, large variations in BDNF, NT-3, NT-4, TrkB and TrkC expression were observed, with no specific correlation with cell size or fast vs. slow motor unit composition (Coprav et al., 2000). The heterogeneity in neurotrophin mRNA expression could be related, at least in part, to differences in muscle activation history between hindlimb muscles and unrelated to motor unit type or cell size. It is presently unknown if fibers in mixed muscles such as the DIAm display differential expression of neurotrophins or their receptors.

The coordinated postnatal development of all components of DIAm motor units (Mantilla et al., 2008) suggests that studying the mechanisms underlying muscle fiber growth and/or fiber type specialization may yield important clues to the origin and maintenance of motor unit diversity. For example, NRG-1 may constitute a nerve-derived trophic influence for muscle fiber growth during perinatal development. NRG-1 is expressed by motor neurons during embryonic and early postnatal development (Sandrock et al., 1997), and induces acetylcholine receptor (AChR) expression at embryonic and perhaps adult neuromuscular junctions likely via its effect on Schwann cells (Jo et al., 1995). In addition, NRG-1 induced phosphorylation of ErbB receptors results in downstream activation of the PI3K/Akt intracellular pathway, which is a critical regulator of muscle protein synthesis and hypertrophy (Bodine et al., 2001). All members of the ErbB receptor family can signal through PI3K, and the ErbB2/ErbB3 heterodimer is a very strong activator of PI3K given the multiple consensus sites on ErbB3 (Hellyer et al., 1998; Citri et al., 2003). Indeed, NRG-1 induces protein synthesis in the postnatal DIAm (Hellyer et al., 2006). It is currently unknown whether there are differences in NRG subtypes or ErbB receptor expression during development or across motor unit types, and whether NRG signaling contributes to the differential postnatal growth of DIAm fibers.

Although most studies have examined NRG-1 effects on neuromuscular junction formation (Sandrock et al., 1997; Buonanno et al., 2001; Sanes et al., 2001), other members of the NRG family are likely involved. In mice with tissue-specific deletions of NRG-1 expression (obtained using the CRE recombinase system), inactivating NRG-1 in motor neurons, muscle fibers or both did not prevent AChR clustering (Jaworski et al., 2006). In agreement, mice lacking skeletal muscle ErbB2 and ErbB4 receptors displayed normal neuromuscular junction development and maintenance (Escher et al., 2005). Both motor neurons and terminal Schwann cells express NRG-2, which is localized around perisynaptic Schwann cells at neuromuscular junctions (Rimer et al., 2004). In addition, NRG-2 induced AChR transcription in cultured myotubes expressing ErbB4 either singly or in combination with ErbB2 or ErbB3, but not in myotubes expressing only ErbB2 and ErbB3. Taken together, these results highlight the complexity of determining unique effects of a specific trophic factor family in vivo. The seemingly redundant expression of trophic factors (e.g., NRG-1 and NRG-2) and receptor isoforms (e.g., ErbB2, ErbB3 and ErbB4) makes unequivocal interpretation of trophic effects difficult when exogenous factors are administered or when expression of a single trophic factor or receptor is mutated or down-regulated.

5. Role of trophic factors in motor unit plasticity

In a series of studies, Mitchell and colleagues examined the role of neurotrophins and their receptors in the plasticity of phrenic motor output (Kinkead et al., 1998; Johnson et al., 2000; Baker-Herman et al., 2004; Golder et al., 2008). Cervical dorsal rhizotomy augments the

serotonin-dependent long-term facilitation induced by intermittent hypoxia (Kinkead et al., 1998), and increases BDNF and NT-3 expression in the cervical spinal cord with no change in GDNF expression (Johnson et al., 2000). Intermittent hypoxia-induced long-term facilitation of phrenic motor output requires new BDNF synthesis in the spinal cord, and application of BDNF to the cervical spinal cord is sufficient to elicit a similar change in phrenic motor output (Baker-Herman et al., 2004). In addition, transactivation of TrkB receptors induced by adenosine 2a receptor ligands is sufficient to elicit facilitation of phrenic motor output (Golder et al., 2008). Importantly, the exact cellular substrate(s) for these forms of phrenic motor neuron plasticity are still unclear. Whether increased neurotrophins derive from motoneurons exerting an autocrine effect on TrkB receptors present on the motor neurons themselves, or act indirectly on neighboring cells to augment neurotrophin release (Canossa et al., 1997) or induce production of other factors (Reichardt, 2006) deserve further investigation.

Neurotrophins have important synaptic effects, even in the adult. For instance, neurotrophins can directly modulate synaptic efficacy (Kang et al., 1995; Kafitz et al., 1999), and neurotrophin synthesis and release are regulated by neuronal activity in the hippocampus (Schinder et al., 2000; Poo, 2001). BDNF may stimulate synapsin I phosphorylation via activation of a MAPK pathway (Jovanovic et al., 2000), and therefore, regulate neurotransmitter release (Hilfiker et al., 1999). In adult rat DIAM-phrenic nerve preparations, the neurotrophins BDNF and NT-4 improved neuromuscular transmission, likely via TrkB activation (Mantilla et al., 2004b). Isometric contractile properties of the DIAM were unaffected by either neurotrophin treatment or TrkB inhibition, suggesting a presynaptic effect of BDNF and NT-4 on neuromuscular transmission. In agreement, BDNF potentiates both spontaneous and evoked synaptic activity in cultured amphibian neuromuscular junctions (Lohof et al., 1993), an effect that is greatly facilitated by presynaptic depolarization (Boulanger et al., 1999). Neuromuscular junctions formed onto amphibian myocytes overexpressing NT-4 showed 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 et al., 1997). Taken together, these results provide converging evidence for an important presynaptic effect of BDNF and NT-4 acting via TrkB receptors on regulation of synaptic vesicle release and neuromuscular transmission. In cultured amphibian neuromuscular junctions, NT-3 also potentiates postsynaptic currents, acting via PI3K, phospholipase C- γ and increased inositol 1,4,5-triphosphate (IP3)-induced Ca^{2+} release (Lohof et al., 1993; Yang et al., 2001). Whether neurotrophins have different effects depending on motor unit type has not been directly explored, but it is tempting to speculate that neurotrophins might underlie fiber type differences in synaptic efficacy at the neuromuscular junction (Mantilla et al., 2004a; Ermilov et al., 2007; Rowley et al., 2007).

GDNF may play an important role in neuromuscular junction plasticity both during development and in the adult. Anterogradely transported GDNF modulates presynaptic vesicle release, increasing spontaneous neurotransmitter release in neonatal mouse neuromuscular junctions (Ribchester et al., 1998). In transgenic mice overexpressing GDNF, axonal branching of motor neurons is increased (Nguyen et al., 1998). Furthermore, exogenous administration of GDNF during the first postnatal week increased neuromuscular junction remodeling, reducing synapse elimination, and thus increasing innervation ratio and motor unit size (Keller-Peck et al., 2001). In adult rodents, muscle activity may influence GDNF expression. For example, treadmill training increased GDNF content whereas hindlimb unloading decreased GDNF in both rat soleus and gastrocnemius muscles (Wehrwein et al., 2002). The magnitude of changes in GDNF expression was similar across these muscles, suggesting that motor unit type might not be as important in determining GDNF expression. However, the level of activity of these hindlimb muscles is low when compared to respiratory muscles such as the DIAM (Kong et al., 1986; Hensbergen et al., 1997). It is currently unknown if motor unit type differences in GDNF expression are present in the DIAM.

Whether multiple trophic factors (e.g., BDNF and GDNF) modulate neurotransmitter release at individual synapses is presently unknown and clearly deserves to be explored. It is tempting to speculate that these trophic factors exert non-overlapping functions related to motor unit type rather than truly redundant effects, but this hypothesis has not been examined directly.

6. Role of trophic factors in neural regeneration

6.1. Nerve injury

Neurotrophin effects on motor neuron survival and axon regeneration following peripheral nerve injury have been studied extensively (Sakuma et al., 2001; Mousavi et al., 2002; Boyd et al., 2003). In neonatal rats, sciatic nerve axotomy leads to atrophy of denervated muscles including the soleus, tibialis anterior and EDL muscles with associated motor neuron loss and reduced muscle fiber number and cross-sectional area. Fibers expressing MHC_{2B} seem particularly susceptible to axotomy (Mousavi et al., 2002). Administering BDNF, NT-3 or NT-4 in combination with CNTF at the site of nerve transection reduces motor neuron loss and prevents axotomy-induced muscle atrophy (Mousavi et al., 2002; Mousavi et al., 2004). In postnatal rats, loss of MHC_{2B}-expressing muscle fibers in the EDL and tibialis muscles is prevented by BDNF (Mousavi et al., 2004), but not by NT-3 or NT-4 (Mousavi et al., 2002). In adults, NT-4 supports type S motor units in the soleus muscle, preventing muscle atrophy and muscle fiber loss (Simon et al., 2003). Interestingly, it seems that neurotrophins both promote and inhibit axon sprouting depending on the receptor they activate. In particular, neurotrophin-induced p75-activation inhibits axon sprouting of axotomized adult motor neurons, whereas TrkB activation promotes neurite outgrowth (Boyd et al., 2003). Indeed, BDNF or NT-4 may facilitate and inhibit axon outgrowth when administered at low and high doses, respectively, given the different neurotrophin affinity of these receptors (Reichardt, 2006). Neural regeneration post-nerve injury may thus depend not only on the availability of specific neurotrophins (e.g., NT-4 for type S or BDNF for type FF motor units), but also on the balance of receptors expressed across motor unit types. Further studies are needed to clarify the possible roles of neurotrophins in the survival of motor neurons and muscle fibers both during development and in the adult, and specifically in models using muscles of mixed motor unit composition such as the DIAM.

Members of the GDNF family of trophic factors do not seem to play a role in presynaptic sprouting post-nerve transection. Interestingly, skeletal muscles express both GDNF and neurturin (Henderson et al., 1994; Nguyen et al., 1998; Suzuki et al., 1998). GDNF mRNA expression is increased in partially denervated human skeletal muscle obtained from patients with peripheral neuropathy or amyotrophic lateral sclerosis (Lie et al., 1998). However, in a recent study, mice with a targeted deletion of RET kinase activity in cranial motor neurons displayed normal hypoglossal motor neuron sprouting and neuromuscular junction re-innervation following axotomy (Baudet et al., 2008). Exogenous treatment with GDNF increased the number of motor axons at neuromuscular junctions only when administered during an early developmental time window (first postnatal week), not when treatment was begun afterwards (Keller-Peck et al., 2001). These results highlight a possible developmental role of GDNF on axon sprouting, in contrast to a more limited role in the adult. However, further studies are needed, specifically with quantification of GDNF and neurturin levels in Schwann cells and muscle fibers, as well determination of GFR α 1-4 receptor expression in axon terminals.

NRGs may have a complex role in supporting motor units following nerve or muscle injury. NRG-1 isoform expression is reduced in facial motor neurons post-axotomy, gradually returning to control levels as re-innervation occurs (Kerber et al., 2003). However, mice with a specific ErbB2 deletion in adult Schwann cells (obtained using an inducible CRE recombinase system) do not display deficits in Schwann cell proliferation following sciatic

nerve transection (Atanasoski et al., 2006), suggesting that NRGs may not directly regulate nerve repair in the adult. It is not clear if a minimum level of NRG/ErbB signaling in Schwann cells is necessary for axon sprouting and myelination following nerve injury. With bupivacaine injection into the tibialis anterior muscle in adult rats, muscle NRG-1 expression decreased initially (Hirata et al., 2007). After 4-6 days, NRG-1 mRNA and protein levels gradually increased, being immunohistochemically localized to differentiating satellite cells. Importantly, spinal cord NRG-1 protein (not mRNA) levels showed a similar temporal expression profile post-injury. Taken together, these results suggest that following muscle injury, regenerating satellite cells increase their expression of NRG-1 which can then act both on neighboring muscle cells to promote muscle fiber growth and on motor neurons (likely via retrograde transport) to promote motor neuron survival and outgrowth (sprouting) of spared axons. These possibilities remain to be explored explicitly. Additional studies are also needed to determine the signaling pathways induced by NRG-1 to promote muscle and neural regeneration post-injury.

6.2. Spinal cord injury

A number of studies have reported changes in the expression of trophic factors such as NGF, BDNF, NT-3, NT-4 and GDNF following spinal cord injury (Bennett et al., 1999; Dougherty et al., 2000; Satake et al., 2000; Widenfalk et al., 2001). Most of these studies examined mRNA expression rather than protein expression, and limited temporal expression profiles. For instance, after mechanical injuries of the midthoracic spinal cord in adult rats (either via compression or complete transection), GDNF mRNA expression increased in meningeal cells and glia at the injury site, whereas p75 mRNA increased in neurons and surrounding microglia (Widenfalk et al., 2001). More prominently, GFR α 1 and truncated TrkB receptor mRNA expression increased throughout the spinal cord white matter by 6 weeks post-injury, being localized primarily to astrocytes (Widenfalk et al., 2001). Following a midthoracic compression injury, the number of astrocytes and microglia displaying BDNF immunoreactivity increased over time at the injury site but not at more distant sites (Dougherty et al., 2000; Ikeda et al., 2001). Detailed, systematic examinations of motor neuron expression of trophic factors or their receptors are not available, especially for motor neurons in pools below the level of injury. Such studies would be particularly meaningful in the case of phrenic motor neurons given that these motor neurons receive excitatory respiratory drive primarily from medullary premotor neurons (Feldman et al., 1985; Ellenberger et al., 1990; Dobbins et al., 1994; Yates et al., 1999). Importantly, comparisons across experimental models (e.g., cervical vs. thoracic levels, mechanical vs. traumatic injury), animal species, and timing of the measurements is complicated by differences in the cellular substrates and even the direction of the change in trophic factor expression post-injury.

Understanding the cellular substrates involved in post-spinal injury changes in expression of trophic factors or their receptors seems critical to the development of rational therapeutic interventions that would enhance functional recovery. Several studies showed that delivery of combinations of trophic factors (either via chronic infusion, adenoviral transfection or stem cell grafts) increases motor neuron survival and axon sprouting within the injured cord (Bregman et al., 1997; Novikova et al., 2002; Lu et al., 2003; Ruitenberg et al., 2004; Iarikov et al., 2007). Unfortunately, increased neuronal survival or axonal sprouting does not readily translate into functional recovery. In recent studies, significant reorganization of spinal cord circuits involved in locomotor behaviors was observed following spinal cord injury (Cai et al., 2006; Courtine et al., 2008) and was enhanced by a number of interventions including BDNF, NT-3 or GDNF treatment (Dolbeare et al., 2003; Iarikov et al., 2007). Whether respiratory circuits involved in the control of DIAM activity undergo similar reorganization post-injury and whether trophic factors promote recovery of rhythmic respiratory activity have not been studied to date.

Another aspect that deserves further attention is that of trophic support of cells in the vicinity of motor neurons, which may thus indirectly affect motor unit properties. For example, motor neuron-derived NT-3 serves as a survival factor for spinal interneurons (Bechade et al., 2002). In this study, a p75-conjugated immunotoxin was used to induce motor neuron loss, which then increased apoptosis of interneurons in organotypic cultures of embryonic rat spinal cord. Exogenous application of NT-3 rescued interneurons from apoptosis, whereas quenching NT-3 with a soluble TrkC receptor induced even greater interneuron loss (Bechade et al., 2002). Conversely, pro-NGF secreted by astrocytes promotes motor neuron apoptosis via activation of the p75 receptor (Domeniconi et al., 2007). The p75 receptor is expressed in spinal motor neurons primarily up to the second post-natal week, following which its expression is normally downregulated (Yan et al., 1988). However, motor neuron expression of p75 increases following both spinal cord injury (Widenfalk et al., 2001) or peripheral nerve injury (Koliatsos et al., 1991; Xie et al., 2003), possibly rendering adult motor neurons susceptible to p75-induced apoptosis again. Complex trophic interactions likely occur within the spinal cord in the environment surrounding phrenic motor neurons, where motor neuron-derived trophic factors can affect presynaptic terminals, interneurons and surrounding glial cells. Trophic factors from any of these sources can also directly exert both positive and negative effects on motor neurons depending on the specific context of trophic factor receptor expression post-injury.

7. Summary/Conclusions

The DIAM is a highly active muscle, with a duty cycle in most species ~35% (Kong et al., 1986; Sieck, 1988; Butler et al., 1999), and must be active from birth onwards. Phrenic motor neurons provide innervation to the DIAM and show remarkable diversity in functional properties. Given this remarkable and unique activation history, DIAM motor units may be particularly responsive to trophic influences and/or inactivity. Most studies have used immunohistochemical methods and in situ hybridization to localize trophic factor expression to large neurons in the ventral spinal cord (including cervical segments). Other studies have determined protein and mRNA expression in ventral horn of the cervical spinal cord. Clearly, these results need to be interpreted with caution when extending the findings to specific cellular substrates within the phrenic motor nucleus. Future studies using novel techniques, e.g., laser capture microdissection where retrogradely-labeled phrenic motor neurons can be uniquely assessed for mRNA measurements, hold great promise in providing cell-based measurements of trophic factor or receptor expression. Detailed and accurate knowledge of the network of trophic interactions at all levels of the motor unit might provide new directions in the study of respiratory motor control.

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Figure 1.

Schematic representation of a motor unit (i.e., a motoneuron and the muscle fibers it innervates) and the potential sources of trophic factor influence on motor units. Trophic factors and their receptors (in *italics*) are noted for each source. A ? sign is used if expression at that site is not reported consistently. *, denotes expression mainly following injury or disease. Parentheses indicate expression by only a subset of motor neurons. See text for details. BDNF, brain-derived neurotrophic factor; GDNF, glial cell line-derived neurotrophic factor; GFR, GDNF family receptor; NRG, neuregulin; NT, neurotrophin; p75^{NTR}, p75 neurotrophin receptor; RET, rearranged in transfection receptor; Trk, tropomyosin-related kinase receptor.