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GDNF Content and NMJ Plasticity in Slow and Fast Twitch Myofibers Follows Recruitment in Exercise

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by

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Glial cell-line derived neurotrophic factor (GDNF) supports and maintains the neuromuscular system during development and through adulthood by promoting neuroplasticity. GDNF may play a role in delaying the onset of aging and help compress morbidity by preventing motor unit degeneration. Exercise has been shown to alter GDNF expression differently in slow and fast twitch myofibers. The aim of this dissertation project is to determine if different intensities of exercise can promote changes in GDNF expression and neuromuscular junction (NMJ) morphology in slow and fast twitch muscle fibers. Skeletal muscle fibers were analyzed from adult Sprague Dawley rats aged 4 weeks old and 6 months old. The phenotype of the skeletal myofibers analyzed are from predominantly slow motor units (soleus; SOL) and fast motor units (extensor digitorum longus; EDL and plantaris; PLA). Animals were exercised through voluntary and involuntary means in a swimming barrel and a running wheel with and without resistance for two weeks. The intensity of exercise was altered by different modes of exercise as well as different running speeds and with the use of resistance. GDNF protein content was determined by enzyme-linked immunosorbant assay and immunohistochemistry was utilized to determine myofiber size, end plate measurements, and localization of GDNF. GDNF protein content was increased ($P < 0.05$) in all

recruited myofibers including; slow twitch myofibers (SOL) following all training protocols and fast twitch myofibers (PLA) following higher intensity exercise such as running at speeds faster than 30m/min. Although not significant, GDNF increased 60% in fast twitch EDL myofibers following swim-training. A relationship exists between GDNF protein content and end plate area. These results indicate that GDNF protein content is upregulated in skeletal muscle fibers that are recruited to meet the demands of the given task, in an activity-dependent manner, and induce neuroplasticity at the neuromuscular junction. These findings help to inform exercise prescription to preserve the integrity of the neuromuscular system through aging, injury, and disease.

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CHAPTER I

INTRODUCTION

The broad goal of the research in our laboratory is to explore what factors regulates glial cell line-derived neurotrophic factor (GDNF) protein expression in the neuromuscular system and what plastic changes are observed in the peripheral nervous system following altered GDNF expression.

Skeletal Muscle Mass

Skeletal muscles show enormous plasticity to adapt to stimuli and have been subject to past and continuing selection to best match their structure and function to their environment. Muscles have been modified to meet and respond to particular demands encountered in order to optimize their effectiveness in terms of energy consumption, generation of short-term maximum force or continuous low level of force (Gans & Gaunt, 1991). Skeletal muscle mass and myofiber size, therefore, vary according to physiological, pathological, and environmental conditions. Skeletal muscle mass is defined as the mean volume of the myofibers (fiber length X fiber cross-sectional area (CSA)) and the number of fibers present in a given skeletal muscle. Mature fibers only change in response to conditions causing muscle hypertrophy or atrophy and the number of fibers in a muscle rarely increase (Gollnick, Timson, Moore, & Riedy, 1981). Therefore changes in the mature myofiber usually only occur through a change of the CSA or a loss in the number of fibers, with CSA being the main cause between 20 and 50 years of age (Lexell, Taylor, & Sjöström, 1988).

Muscle Hypertrophy

An increase in skeletal muscle mass and fiber CSA or hypertrophy, occurs during development and in response to functional overload (e.g. strength training and ablation of synergistic muscles) or by means of anabolic hormonal stimulation (e.g. testosterone or β_2-adrenergic agonists). Muscle hypertrophy occurs following increased protein synthesis via increases in transcription, translation or through the addition of myonuclei (Bodine, 2006). The two fundamental adaptations necessary for muscle hypertrophy are increased protein synthesis and satellite cell proliferation. A progressive and continuous increase in muscle mass has been shown in response to functional overload with the speed of development depending on the mode of training, the muscle group involved, and the initial training status (Blazevich, Gill, Bronks, & Newton, 2003; Higbie, Cureton, Warren, & Prior, 1996; Men, Young, Stokes, & Crowe, 1985; Narici, Roi, Landoni, Minetti, & Cerretelli, 1989). Muscle hypertrophy has been associated with significant changes in muscle architecture, including increases in fascicle length (in series) and pennation angle (in parallel) (Gans & De Vree, 1987). These adaptations increase the addition of sarcomeres within a single fascicle and therefore, increase the maximal force production. It is well understood that the remodeling of the muscle architecture is most stimulated following skeletal muscle stretch combined with overload (Goldspink, 1999). High intensity and resistance training have been used for decades as a means to induce overload to the muscles, recruit fast twitch myofibers and induce compensatory hypertrophy.

Resistance Training

Resistance training is known to increase protein synthesis under conditions of increasing loading and during the recovery phase resulting in compensatory growth of skeletal muscle (Koopman & van Loon, 2009; Miyazaki & Esser, 2009). Compensatory hypertrophy occurs in response to active cross-bridge interactions (concentric, eccentric or isometric contractions), a gravity-based environment, or via external application of force (passive stretch) and can occur in the absence of circulating pituitary hormones or insulin (Goldberg, 1967). Mechanical loading in skeletal muscle can induce increased expression of insulin-like growth factor-1 (IGF-1), which signals through the PI3K/Akt (serine/threonine kinase) pathway, leading to increases in protein synthesis and skeletal muscle hypertrophy in vitro and in vivo (Adams & McCue, 1998; Rommel et al., 2001). More recently, it has been found that hypertrophy can be induced without the downstream signaling of IGF-1 and that in its absence, a mammalian target of rapamycin (mTOR) signaling pathway can stimulate muscle mass growth (Spangenburg, Le Roith, Ward, & Bodine, 2008). Skeletal muscle mass and cross-sectional area of the myofibers have been found to hypertrophy rapidly and by more than twofold as early as 2-3 weeks following activation of Akt (Lai et al., 2004). Detectable changes in muscle size has also been reported as early as three weeks post resistance training when observed macroscopically (ultrasonography) (Seynnes, de Boer, & Narici, 2007), providing additional evidence that muscle hypertrophy may be providing strength gains rapidly following overload.

Muscle Atrophy

Skeletal muscle atrophy can be defined as a wasting or decrease in muscle mass most often in response to reduced contractile activity, reduced mechanical load, or modified environmental conditions. Atrophy is most often associated with aging, poor nutrition/starvation and chronic diseases resulting from genetic abnormality or systemic disease (McKinnell & Rudnicki, 2004). Regardless of its etiology, atrophy is characterized by a size reduction or loss of myofibers as well as reductions in force generation, total protein content, and fatigue resistance (Jackman & Kandarian, 2004). The balance of dynamic anabolic and catabolic reactions determines the level of muscle protein and maintains skeletal muscle. Atrophy occurs when the rates of protein degradation exceed those of protein synthesis leading to decreases in total muscle protein, muscle mass and impaired functional ability. Neural stimulation is one of the most important determinants of gene expression in skeletal muscles, determining the myofiber genotypic and phenotypic characteristics (Fluck & Hoppeler, 2003). Atrophy follows reduced neural stimulation and has been modeled in hindlimb suspension, immobilization, nerve transection or denervation. Progressive denervation is often associated with aberrant re-innervation, aging and neuromuscular disorders. The progressive and continual loss of functional motor neurons limits the capacity to sprout and regenerate denervated muscle tissue leading to diminished muscle mass and strength, referred to as muscle quality or sarcopenia.

Muscle Quality

Skeletal muscle quality includes both skeletal muscle mass and strength and has been studied extensively in the aging population with consistent findings of 30-50% decrease in skeletal muscle mass in both men and women between the ages of 40-80 years, with a rate of loss of 1.0-1.5% per year after the age of 60 (Akima et al., 2001; Men et al., 1985; Vandervoort, 2002). Accompanying muscle mass loss is a decrease in strength, power and fatigue resistance (Frontera, 2008; Goodpaster et al., 2006; Men et al., 1985; Skelton, Greig, Davies, & Young, 1994). The loss of strength and power has largely been attributed to the preferential loss of large fast motor units through the natural aging process and as a result of injury or degenerative neuromuscular diseases.

Motor Units

A motor unit is defined as a motor neuron and the family of myofibers that it innervates. The neuromuscular junction (NMJ) provides the sole link between the motor neuron and skeletal muscle fibers. The NMJ comprises a presynaptic apparatus (nerve terminal), a postsynaptic apparatus (specialized portion of the myofiber) and perisynaptic Schwann cells (PSC) that extend processes close to the nerve terminals with finger-like extensions into the synaptic cleft (Auld & Robitaille, 2003).

Motor units are classified based on the mechanical and fatigue properties of the motor neuron and innervated muscles fibers, which are precisely matched within one given motor unit (Burke, Levine, Tsairis, & Zajac III, 1973). Motor units exhibit considerable functional diversity in size, mechanical and fatigue properties (Burke et al., 1973). There are four commonly described classifications of motor units with

corresponding expression of specific myosin heavy chain isoforms in the myofibers as follows; 1) slow, fatigue resistant (type I fibers-$MyHC_{slow}$), 2) fast, fatigue resistant (type IIa fibers-$MyHC_{2a}$), 3) fast, fatigue-intermediate (type IIx fibers-$MyHC_{2x}$), and 4) fast, fatigable (type IIb fibers-$MyHC_{2b}$). They are arranged on a continuum in which slow motor units are the smallest, produce the least amount of force, are the most fatigue resistant and are recruited first. On the other end of the continuum are the fast motor units, which are the largest, produce the most force, are the least fatigue resistant, and are recruited last. The innervation ratios vary across muscles, but within a single muscle, the ratios are usually greater for fast motor units when compared to slow motor units, aiding to a greater force production. The recruitment pattern follows the Henneman's principle (Henneman, 1981) in which the small motor units are the first and most often recruited during activities requiring low intensity, such as sitting, standing, or walking and the large motor units are recruited only during higher intensity activities when more force is needed to execute the task or skill such as running, swimming and jumping.

Denervation

The motor unit undergoes significant changes during the natural aging process and as a result of selective neuromuscular diseases. Age-related changes occurring at the NMJ include withdrawal of nerve terminals from innervated myofibers, ultra-terminal sprouting, re-innervation of denervated myofibers by surviving motor neurons, greater branching complexity, expanded postsynaptic endplate dimensions, a conversion to slow type myofiber phenotypes, and decreased size of end plates.

Motor unit losses of 25% in young verse old (20 vs. 90 years) humans (Tomlinson & Irving, 1977) and a 30-40% decline in adult verse old animals have been observed (L. Edström, 1987; L. J. Einsiedel, 1992). The loss appears to occur in a specific ordered sequence according to the size of the motor unit. The fast motor units are the first and the most susceptible to undergo denervation and atrophy followed by fast intermediate and finally the smallest and most plastic and resilient; slow motor units (Chai, Vukovic, Dunlop, Grounds, & Shavlakadze, 2011; E. Edström et al., 2007; Frey et al., 2000). A 2.5 fold increase of fully denervated endplates in the large motor units has been observed with no changes in the small motor units through the aging process (Chai et al., 2011). The sparing of the small motor units has been thought to occur in order to be able to continue slow type activities such as standing and postural stability critical for the aging adult (Deschenes, Roby, Eason, & Harris, 2010). In this way, the sparing of denervation is thought to be reserved for those muscles that are actively recruited through neuromuscular activity.

The loss of the fast motor units leaves some fast myofibers without any innervation. Some of these fibers get incorporated into remaining motor units by axonal sprouting (Frey et al., 2000). The re-innervation of fast twitch myofibers by intermediate or slow motor units result in decreased power and strength production observed in the aging animal.

This re-innervation compensation increases the endplate area per fiber ratio (innervation ratio), thus increasing the size of the remaining motor units (BaliceGordon, 1997; Gutmann, 1971; Ludatscher, 1985; Rosenheimer, 1990). It has been proposed that

the re-innervation overburdens and exhausts the remaining intermediate and slow motor units, progressively leading to further denervation. Fibers that do not become re-innervated undergo denervation atrophy and are eventually lost. This motor unit loss eventually causes disability through muscle weakening and functional denervation, morbidity, and mortality (Frey et al., 2000; Hegedus, Putman, Tyreman, & Gordon, 2008). Studies observing gene expression following denervation also support these observed plastic changes in the motor unit. Substantial changes in gene expression were observed among ~2,000 genes monitored following a short period of two weeks post denervation (Raffaello et al., 2006). The hindlimb fast twitch muscle, tibialis anterior, showed clear signs of a transition from fast to slow phenotype with decreased $MyHC_{2B}$ mRNA and an increase in $MyHC_{2A}$ mRNA. The transition to a slower phenotype is understood as a result of a gradual replacement of fast isoform $MyHC_{2B}$ by $MyHC_{2A}$ through the intermediate $MyHC_{2X}$ (Pette & Staron, 2001; Schiaffino & Reggiani, 1996) with a substantial increase in $MyHC_{slow}$ expression observed 5 weeks post denervation (Midrio, Danieli-Betto, Megighian, & Betto, 1997). The shift to a slower phenotype is accompanied by a prolonged time to peak force, a leftward shift of the force-frequency curve and reduced metabolic activity (Raffaello et al., 2006). It has been shown that denervation precedes and is a necessary pre-requisite to the loss of muscle mass and fiber type conversion that characterizes sarcopenia (Deschenes et al., 2010). Therefore, it has been postulated that activation of the fast motor units through exercise could be implemented to slow or prevent the first steps in the denervation process.

The recruitment of fast twitch myofibers occurs during higher intensity exercise and far less often than those postural slow twitch myofibers, especially with increased sedentary behaviors. Recruitment of skeletal muscle, however, can maintain the NMJ integrity in slow and fast twitch myofibers through the aging process (Deschenes et al., 2010). Recruitment of skeletal muscle through exercise has, therefore, been an active area of research in an effort to slow or reverse the aging process of the neuromuscular system (Deschenes et al., 2010; Kanda & Hashizume, 1998).

Rapid cellular and molecular changes follow denervation of a target tissue. Motor neurons must undergo significant morphological, physiological and molecular changes in order to reinnervate a target skeletal muscle.

Re-innervation

Axonal sprouting is a natural compensatory mechanism that attempts to reinnervate target skeletal muscle following denervation. In the axonal sprouting process, fine nerve processes or sprouts grow out from the intact axons at either the motor endplates to give rise to ultraterminal sprouts or the nerve terminal to give rise to preterminal sprouts or the nodes of Ranvier to give rise to nodal sprouts. Axonal sprouting may compensate for losses up to 85% of their normal number of motor units and are restricted beyond a 3-8 fold increase (Brown, Holland, & Hopkins, 1981; Gordon, Hegedus, & Tam, 2004; Rafuse & Gordon, 1996). Beyond this number, axonal sprouting fails to reinnervate all denervated myofibers and weakness ensues (Gordon et al., 2004; Tam & Gordon, 2003).

To begin axonal sprouting, perisynaptic Schwann cells engage in an active and critical role by rapidly converting from a mitotically quiescent myelinating phenotype to a rapidly proliferating non-myelinating phenotype (Fu, 1997). Proliferating Schwann cells form linear bands within the endoneurial sheath at both the innervated and denervated endplates, known as Bands of Büngner, which act as a tunnel-like bridge to lead and guide the growth of axonal sprouts and reinnervate denervated endplates (Son & Thompson, 1995a; Son & Thompson, 1995b; Tam & Gordon, 2003).

Blockade to the pre-and post-synaptic apparatuses of a partial denervated muscle have shown reduced bridging of the presynaptic Schwann cell processes between innervated and denervated endplates (Love & Thompson, 1999). In addition, it has been shown that inactivity caused by blocking postsynaptic (α-bungarotoxin) and presynaptic (TTX) neuromuscular activity reduced motor unit enlargement and axonal sprouting in large and small motor units following partial denervation (Connold & Vrbova, 1991). Increased neuromuscular activity by prolonged treadmill exercise showed increased sprouting and increased force production by slow and fast motor units in partially denervated skeletal muscle (L. J. Einsiedel & Luff, 1994). It was postulated that the fast motor units increased mean maximal tetanic force and mean estimated innervation ratios of the entire fast motor unit population because those myofibers were recruited during the exercise protocol. Collectively, these studies show that neuronal activity is an important factor in determining the rate at which motor neurons extend axonal sprouts, which help match the functional requirements of a motor unit to the ability to innerve neighboring myofibers. In addition, the preservation of the motor unit including size, innervation,

regenerative response and axonal sprouting, has been shown to largely rely on the availability of neurotrophic factors.

Neurotrophic Factors

Neurotrophic factors are a family of small secreted polypeptides that act as growth factors for survival and phenotypic differentiation of developing neurons as well as maintenance and protection of mature and injured neurons in the adult vertebrate nervous system. Neurotrophic factors are produced by local or target tissues and when neurons fail to obtain a sufficient quantity, neuronal degeneration (loss or atrophy) occurs and apoptotic mechanisms are activated as a result of induced transcriptional processes (Altman, 1992; Yuen, Howe, Li, Holtzman, & Mobley, 1996). A reduction in neurotrophic support, due to genetic background, aging or other factors has contributed to neurodegeneration and disease. The potential of neurotrophic factors to protect aging, injured or diseased neurons from atrophy or apoptosis by restoring and maintaining neuronal function gives them a potential therapeutic role in delaying the aging process, reversing neuronal injury, and preventing or slowing neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, Huntington's disease or amyotrophic lateral sclerosis.

Neurotrophic factors are more complex than what was once described as the original "neurotrophic factor hypothesis" (Yuen et al., 1996). This hypothesis describes neurons as acquiring neurotrophic factors from target-derived signaling, but in addition, we now know that neurotrophic factors can also be synthesized within the neuron (autocrine signaling) or attained from local sources (paracrine signaling) such as glial

cells. In addition, an individual population of neurons can respond to many different neurotrophic factors, more than one neurotrophic factor can be synthesized by the same source, multiple receptors can bind multiple factors, and a synergy may exist among neurotrophic factors that result in a shared outcome (Yuen et al., 1996).

Neurotrophic factors are classified according to their structural and functional similarities. The families of neurotrophic factors include 1) nerve growth factor (NGF)-superfamily; 2) non-neuronal growth factor-superfamily; 3) neurokine or neuropoietin superfamily; and 4) glial cell line-derived neurotrophic factor (GDNF) family.

GDNF

The GDNF family includes GDNF and three structurally related members called neurturin, artermin, and persephin. GDNF was first identified in 1993 in embryonic midbrain dopaminergic neurons (Lin, Doherty, Lile, Bektesh, & Collins, 1993), which are cells that degenerate in Parkinson's disease. GDNF was first given attention due to its ability to increase the high-affinity dopamine uptake and promote cell survival and differentiation of dopaminergic neurons. GDNF was characterized as a distant member of the transforming growth factor-β superfamily. Like other members of this family, GDNF is a disulfide-bonded dimeric protein containing seven conserved cysteine residues with a molecular weight of 30kDa and is glycosylated (Lin et al., 1993). The amino acid sequence of rat GDNF is 93% identical to that in humans (Lin et al., 1993). The mature forms of GDNF are cleaved for secretion and expressed in two splice variants, a larger ($GDNF_{633}$) and a truncated form missing a 78-base pair sequence ($GDNF_{555}$) derived from a single RNA (Springer et al., 1995). The expression of these isoforms are

differentially regulated in rat skeletal muscle in which the predominant isoform, $GDNF_{555}$, is present in healthy muscle and $GDNF_{633}$ becomes the predominant isoform following denervation (Springer et al., 1995). This indicates that GDNF may play an important role in reinnervation of skeletal muscle following denervation.

GDNF Signaling

The GDNF family ligands (GFLs) all signal through the c-ret proto-oncogene (RET) receptor tyrosine kinase that are bound to GDNF family receptor-α (GFRα) receptors. GFRα receptors are bound to the plasma membrane by a glycosyl phosphatidylinositol (GPI) anchor (Poteryaev et al., 1999) and are thought to have three globular cysteine-rich domains with the second domain being most commonly bound by GFLs, which is crucial for RET binding (Airaksinen, Titievsky, & Saarma, 1999; Peterziel, Unsicker, & Krieglstein, 2001). GFLs express high affinity specificity for one of the four main GFRα receptors (GFRα1-4), although some low-affinity cross talk binding also occurs, to activate the GFRα-RET complex. GDNF binds to GRFα1, NRTN binds to GFRα2, ARNT binds to GFRα3, and PSPN binds to GFRα4. The binding of the GFLs recruits RET to the lipid rafts, promoting phosphorylation and dimerization of tyrosine residues and triggers its association with Src required for downstream signaling (Tansey, Baloh, Milbrandt, & Johnson Jr, 2000).

RET Activation

RET is a single pass transmembrane receptor tyrosine kinase comprised of an extracellular region which forms a multiprotein complex in association with GFRα receptors, a transmembrane region and a cytoplasmic kinase domain that is responsible

for autophosphorylating intracellular tyrosine residues. In order to activate RET, GFLs must bind to its respective GFRα receptor in addition to Ca^{2+} ion binding to one of the four extracellular domains of RET (Anders, Kjær, & Ibáñez, 2001) . GFLs cannot bind directly to RET and require a ligand-binding subunit acting as a coreceptor, GFRα1-4, as previously described. Once phosphorylated, RET's tyrosine residues in the intracellular domain serve as high affinity binding sites for various intracellular signaling proteins in the target cells. GFL-RET signaling increases phosphorylation of Src, MEK, ERK and Akt, indicating Ras-ERK pathway, p38 MAPK, and Src-MEK-PI3K pathway activation (Trupp, Scott, Whittemore, & Ibanez, 1999).

Src kinase recruitment with RET activation is associated with MEK phosphorylation and PI3K signaling, which is required for formation of lamellipodia, essential for neurite outgrowth and is responsible for the survival-promoting effects of motoneurons (Soler et al., 1999). GFLs have been shown to promote motor neuron survival through the activation of PI3-kinase intracellular signaling pathway. Although Ras-ERK-MAPK pathway is not directly involved with motor neuron survival (Soler et al., 1999), its sustained activation through GFL signaling is sufficient for neuronal differentiation, neurite outgrowth (Fukuda et al., 1995) and migration of Schwann cells (Airaksinen et al., 1999).

The PI3K pathway can also phosphorylate Akt, which has been implicated in motor neuron survival and neurite elongation activity both in vivo and vitro (Namikawa et al., 2000). In addition, Akt phosphorylation promotes muscle growth and blocks muscle denervation-induced atrophy (Bodine et al., 2001; Léger et al., 2006; Stitt et al.,

2004). Observations of muscle hypertrophy and protection from denervation-induced atrophy have been shown in adult myofibers of Akt transgenic mice. Akt protects the muscle size by inhibiting the nuclear translocation of the FOXO family of transcription factors, blocking the upregulation of MuRF1 and MAFbx, which mediate myofiber atrophy (Glass, 2011).

GDNF and Receptor Distribution

GDNF, RET, and GFRα1 proteins and mRNAs are widely distributed in motor neurons from the earliest stages of their development (Trupp et al., 1999). GDNF mRNA and protein are found in skeletal muscle, and are heavily concentrated and localized to the postsynaptic apparatus of the NMJ, while RET and GFRα1 mRNA are found in the motor neuron and their protein are concentrated in nerve endings at the presynaptic apparatus of the NMJ (Baudet et al., 2008; Suzuki, Hase, Miyata, Arahata, & Akazawa, 1998). The high co-localization of RET and GFRα1 receptor, along with close proximity to GDNF at the NMJ indicates their important trophic role in shaping and maintaining the neuromuscular synapse.

RET and GDNFα1 receptors have been shown to be preserved in the remaining motor neurons in degenerating ALS disease (Mitsuma et al., 1999), as well as upregulated following nerve injury (Burazin & Gundlach, 1998) and during the natural aging process (Bergman, Kullberg, Ming, & Ulfhake, 1999). Sciatic nerve axotomy increases the expression of GDNF in denervated muscle, GDNF and GFRα1 in Schwann cells, and GDNF, RET and GFRα1 in injured motor neurons (Frostick, Yin, & Kemp, 1998). The uncoupling of GFRα1 and RET in Schwann cells in which RET is not

detectable (Treanor et al., 1996) have led researchers to find alternative signaling for GDNF in cells lacking RET receptors. It has been found that GDNF can signal independently of RET by binding to GFRα1 receptors, stimulating a cytoplasmic GFRα1-associated Src-like kinase activity, and activating downstream signaling pathways (Trupp et al., 1999). Regardless of the receptor complex, GDNF and downstream signaling pathways suggest an immediate trophic need by the degenerating and damaged motor neuron.

GDNF has been found to be upregulated in skeletal muscle following ongoing denervation due to neuromuscular diseases (Grundström et al., 1999; Lie & Weis, 1998) and following axotomy and transection of the motor neuron (Burazin & Gundlach, 1998; Naveilhan, ElShamy, & Ernfors, 1997; Trupp et al., 1995). Aging has also increased the expression of GDNF and its receptors. More than 90% of the motor neurons in young adult rats and aged rats contain GFRα1 mRNA with an increased labeling density in the aged animal (Bergman et al., 1999). Also, the increase in GFRα1 mRNA coincided with GFRα1 protein. Likewise, motor neurons express RET mRNA in 70-80% of young adult animals and more than 90% in aged animals with a 50% increase in labeling density in the aged rat (Bergman et al., 1999). These findings together suggest an important trophic role for GDNF to help support the motor neuron during distressed states, slowing motor neuron denervation accompanying aging, injury and disease.

GDNF and Motor Neuron Protection

GDNF is a target derived neurotrophic factor in that it is produced in target tissues such as skeletal muscle and then bound, internalized and retrogradely transported by both motor and sensory neurons in a specific receptor-mediated manner (Yan, Matheson, & Lopez, 1995). GDNF's receptor, RET, is expressed in all spinal cord motor neurons from the earliest stages of their development influencing motor neurons survival and axonal guidance and growth throughout the lifespan (Trupp et al., 1999). As previously mentioned, RET receptors are localized at the presynaptic nerve terminals and in their absence, deficits in presynaptic maturation during development and loss of endplates postnatally are observed (Baudet et al., 2008). By conditionally deleting RET in cranial motor neurons a continual loss of motor neurons was observed following birth, indicating that RET signaling is important for motor neuron survival developmentally and postnatally. In addition, a 20-40% loss of motor neurons and increased apoptosis compared with wild-type controls have been observed in the absence of GDNF or GFRα (Garces, Haase, Airaksinen, Livet, & Filippi, 2000; Moore et al., 1996).

GDNF has been found to be the most potent trophic factor for motor neuron survival and for preventing degeneration and axonal atrophy (Henderson et al., 1994; R. W. Oppenheim et al., 1995; R. Oppenheim, 2000; Yan et al., 1995; Zhao et al., 2004). GDNF has been shown to save motor neurons from programmed cell death in the cervical, brachial and thoracic regions of the spine as well as facial, hypoglossal and spinal accessory cranial motor neurons (R. Oppenheim, 2000). GDNF has been found to

be 75-, 650-, and 2500-fold more potent in preventing programmed cell death when compared to brain derived neurotrophic factor, ciliary neurotrophic factor, and human cholinergic differentiation factor leukemia inhibitory factor, respectively (Henderson et al., 1994). GDNF was also the only neurotrophic factor able to save 100% of motor neurons following axotomy, while maintaining normal soma size and preventing neuronal atrophy (Henderson et al., 1994; R. W. Oppenheim et al., 1995; Yan et al., 1995). GDNF treatment after motor neuron axotomy showed prevention of cell loss and atrophy in comparison to those left untreated that showed 50% loss of motor neurons and evidence of significant atrophy (R. W. Oppenheim et al., 1995). GDNF was not only able to prevent atrophy, but showed significant hypertrophy of the lesioned motor neuron.

GDNF through RET signaling has an important role not only in motor neuron survival, but for synapse maturation, as close to one-half of the neuromuscular synapses are absent in ret-deficient mice post synapse elimination (Baudet et al., 2008). In this same study, motor neuron terminal sprouting was also found to be compromised in the absence of RET. Further evidence that GDNF/GFRα1/RET signaling develops and maintains the NMJ into adulthood comes from studies injecting or overexpressing GDNF that have resulted in an increase of end plate size and hyperinnervation of the end plates by terminal axonal sprouting (Keller Peck et al., 2001, Nguyen, Parsadanian, Snider, & Lichtman, 1998; Zwick, Teng, Mu, Springer, & Davis, 2001). Equilibrium at the NMJ occurs through GDNF signaling by providing continuous synaptic remodeling equilibrium in which axonal extension keeps pace with axonal retraction by promoting motor neuronal terminal branching throughout the lifespan.

Similar plasticity and protection observed with GDNF/GFRα1/RET activation have also been observed following exercise in the neuromuscular system.

Exercise and Motor Unit Protection

Exercise is potently neuroprotective in the central and peripheral nervous systems. Exercise has been shown to fight against abnormal neuronal loss observed in neurodegenerative disorders including dementia (Rolland, 2012), Alzheimer's disease (Nation et al., 2011) and Parksinson's disease (Goodwin, Richards, Taylor, Taylor, & Campbell, 2008). It has been suggested that exercise may provide a non-invasive, non-pharmaceutical mechanism to protect against the onset of various neuromuscular disabilities and diseases.

Likewise, exercise has shown remarkable neuroprotective effects on the peripheral nervous system. The NMJ can exhibit great plasticity and exercise has been an important tool to maintain the synapse structure and function as well as to improve the recovery from injury and ameliorate degenerative changes. When compared to the aged NMJ, exercise has been shown to increase the pre-and post-synaptic apparatus size at the NMJ (Deschenes et al., 1993), increase the size and degree of branching of presynaptic nerve terminals (Andonian & Fahim, 1987), and increase the quanta release of neurotransmitter, acetylcholine (Dorlöchter, Irintchev, Brinkers, & Wernig, 1991).

High intensity exercise, such as resistance training, elicits significant changes in neuromuscular function in the elderly individuals. Resistance training is capable of increasing maximum motor neuron firing frequency, central muscle activation, rapid

muscle force production, fine motor control, muscle CSA and volume in all fiber types, and is capable of inducing the same relative gain in anatomical muscle size between young and old (Aagaard, Suetta, Caserotti, Magnusson, & Kjær, 2010). Pertinent to the role of denervation, resistance training and maintained physical activity have been shown to preserve the size of fast twitch myofibers when compared to age-matched sedentary controls.

Exercise has been shown to have a preferential effect on promoting the enlargement of fast motor units by increasing the innervation ratio and increasing muscle force in partially denervated skeletal muscle (L. Einsiedel & Luff, 1994; Gardiner, Michel, & Iadeluca, 1984). These findings suggest that exercise could be used as a means to deter or slow the denervation of fast motor units, which is a necessary prerequisite for muscle atrophy and loss, observed with sarcopenia. As mentioned earlier, large motor units innvervating fast twitch myofibers are most vulnerable through the aging process. Studies reveal that myelinated motor neuron axons are reduced in numbers and in diameter in the ventral roots, with an accelerated loss of large-diameter axons (Hashizume, Kanda, & Burke, 1988; Kawamura, O'brien, Okazaki, & Dyck, 1977). There appears to be a critical age threshold in which function becomes impaired, marked by greater motor unit loss than years prior. Although studies have reported an acceleration of motor unit loss past the age of 60 years, it is estimated that the critical threshold is reached between 70-80 years of age (McNeil, Doherty, Stashuk, & Rice, 2005). This is thought to occur when collateral sprouting is no longer able to keep pace with motor neuron denervation. The loss is not only age dependent, but also muscle specific, as slow

motor units are retained much later in life than fast motor units (Dalton, 2008), not reaching the critical threshold until the tenth decade of life (Doherty, 2003).

One potential explanation for the discrepancy between muscle types and their vulnerability to the denervation process is the availability of neurotrophic factors, namely GDNF, to preserve and maintain motor units. Exercise has been shown to help in delaying the onset of denervation and to induce NMJ plasticity in those injured or diseased motor units to retain the integrity of the neuromuscular system. Interestingly, exercise has also been shown to play a vital role in the regulation of neurotrophic factors, namely GDNF, the ligand that serves as a potent neuroprotective factor to preserve, remodel and maintain survival of motor units.

Exercise and GDNF Expression

Researchers have used skeletal muscle activation through physical activity, stretch, electrical stimulation and acetylcholine released in cell culture to help identify what regulates GDNF expression (Table 1). GDNF protein content has been shown to increase following low intensity exercise (McCullough, Peplinski, Kinnell, & Spitsbergen, 2011) and during functional loading (Wehrwein, Roskelley, & Spitsbergen, 2002) in slow twitch myofibers. In contrast, GDNF protein content levels in fast twitch myofibers have been shown to be unaffected or decreased following disuse or low intensity activity (McCullough et al., 2011; Wehrwein et al., 2002). These studies support, therefore, that GDNF acts in an activity-dependent manner for slow twitch myofibers and that it is expressed differently in slow and fast twitch myofibers during

low intensity exercise. As previously described, in order to recruit the phasic response of fast motor units, the workload must exceed that of slow motor unit force production capabilities. If it holds true that regulation of GDNF production is activity-dependent for fast motor units as with slow, a higher intensity exercise, which forces recruitment of fast twitch myofibers, may be able to induce its expression. It is possible that through the recruitment of fast twitch myofibers and the subsequent increase in GDNF protein content, that the denervation process may be delayed or halted in the aged, injured or diseased fast motor unit.

TABLE 1.

Regulation of GDNF Protein Expression

Factor	Effect	Site	Reference(s)
High Velocity Run (>30m/min)	+	PLA	(Gyorkos & Spitsbergen, 2014)
	+	SOL	
Resistance Run (>30m/min)	+	PLA	(Gyorkos & Spitsbergen, 2014)
	+	SOL	
Low Velocity Run (10m/min)	-	EDL	(Gyorkos, McCullough, & Spitsbergen, 2014; McCullough et al., 2011; Wehrwein et al., 2002)
	+	SOL	
Swimming	-	EDL	(Gyorkos et al., 2014)
	+	SOL	
Stretch	None	EDL	(McCullough et al., 2011)
	+	SOL	
Electrical Stimulation (low frequency)	-	EDL	(McCullough et al., 2011)
	+	SOL	
Hindlimb suspension	-	SOL	(Wehrwein et al., 2002)
	+	pectoralis major	
nAChRs activation	-	skeletal muscle cells in culture	(Vianney & Spitsbergen, 2011)
Spinal Cord Injury & Exercise	+	SOL	(Dupont-Versteegden et al., 2004)
	+	spinal cord	(Côté, Azzam, Lemay, Zhukareva, & Houlé, 2011)

Adapted from (Vianney, Mccullough, Gyorkos, & Spitsbergen, 2013)

Table 1. Summary of regulation factors affecting GDNF protein expression.

Specific Aims

The specific goal of this dissertation is to identify the exercise intensity that is necessary to upregulate the expression of GDNF protein in fast twitch myofibers and induce neuroprotective plasticity. Understanding the appropriate exercise prescription that will increase GDNF expression in fast motor units can have profound effects on the neuromuscular system including; survival support during development and through adulthood and increased plasticity of pre- and post-synaptic apparatus to meet the needs of the aged, injured or diseased neuromuscular junction. Therefore, the specific aims outlined below are designed to determine how exercise may regulate GDNF expression and how in turn it may contribute to the plasticity of the neuromuscular system.

Specific Aim 1

The hypothesis for specific aim 1 is that swim training exercise increases GDNF protein content in fast twitch skeletal muscle and promotes plasticity at the neuromuscular junction.

Specific aim 1 is designed to better understand the role that exercise intensity plays in the regulation of GDNF expression in slow and fast twitch myofibers. Low intensity exercise has been shown to alter the expression of GDNF differently in slow and fast twitch myofibers by increasing and decreasing GDNF content, respectively (McCullough et al., 2011). Therefore, it is the aim of these studies to examine the changes in GDNF expression and plasticity at the NMJ in slow and fast twitch fibers following a higher intensity swim-training. By developing these studies, the following questions can be answered;

1. Does swim and/or run training recruit slow- and/or fast-twitch muscle?

2. Are there differences in the expression of GDNF in slow and fast twitch myofibers following different training protocols?

3. Are there differences in the plasticity of the NMJ in slow and fast twitch myofibers following run and swim training?

4. Do changes in GDNF protein content correlate with changes in NMJ morphology?

Specific Aim 2

The hypothesis for specific aim 2 is that high velocity *involuntary* running increases GDNF protein content in both slow and fast twitch myofibers and promotes plasticity at the neuromuscular junction.

Specific aim 2 is designed to better understand the effects of GDNF expression and NMJ plasticity following an increase in the velocity of a run training protocol. It is our goal to control for the velocity of the run by running rats in involuntary running wheels at a high and low velocity and observe any changes within slow and fast twitch skeletal muscles and their respective synapses.

1. Does *involuntary* training recruit slow and/or fast twitch muscle?

2. Can two weeks of *involuntary* exercise alter GDNF protein content in slow and fast twitch skeletal muscle?

3. Are there differences in the plasticity of the NMJ in slow and fast twitch myofibers following *involuntary* exercise?

4. Does a relationship exists between GDNF protein content and changes in NMJ morphology following *involuntary* exercise?

Specific Aim 3

The hypothesis for specific aim 3 is that high intensity *voluntary* exercise increases GDNF protein content in both slow and fast twitch skeletal muscle and promotes plasticity at the neuromuscular junction.

Specific aim 2 is designed to better understand the effects of high intensity voluntary training on GDNF's expression in skeletal muscle and on the morphology of the NMJ. Rats have been shown to exercise at high intensities with short high velocity bursts when allowed to train voluntarily. Exercise with resistance added to a wheel has also been shown to increase the intensity of exercise by inducing hypertrophy of fast twitch myofibers. Therefore it is the aim of this study to examine the effects of voluntary resistance training (slow velocity, high resistance) and voluntary no-resistance training (high velocity, low resistance) on GDNF's expression in both slow and fast twitch myofibers. By developing these studies, the following questions can be answered;

1. Does *voluntary* training recruit slow and/or fast twitch muscle?

2. Can two weeks of *voluntary* exercise alter GDNF protein content in slow and fast twitch skeletal muscle?

3. Are there differences in the plasticity of the NMJ in slow and fast twitch myofibers following *voluntary* exercise?

4. Does a relationship exists between GDNF protein content and changes in NMJ morphology following *voluntary* exercise?

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CHAPTER II

GDNF EXPRESSION AND NMJ PLASTICITY IN SKELETAL MUSCLE FOLLOWING ENDURANCE EXERCISE

Introduction

Maintaining a healthy neuromuscular system requires a continual supply of neurotrophic factors (NFs) to support developing and mature motor neurons. NFs support motor neurons throughout the lifespan by supporting maturation during embryonic life, maintenance through adulthood, and regeneration after injury (Keller-Peck et al., 1997; Keller Peck et al., 2001; Oppenheim et al., 1995; Trupp et al., 1995; Wang et al., 2002; Yan et al., 1995; Zwick et al., 2001). NFs have been shown to act alone and synergistically in their role to promote proper development and plasticity of adult and aged neurons. Of the many NFs identified to date, glial cell-line derived neurotrophic factor (GDNF) has been shown to be the most potent trophic factor for motor neuron survival *in vitro* (Henderson et al., 1994; Lin et al., 1993) and *in vivo* (Henderson et al., 1994). GDNF has been classified as belonging to the transforming growth factor superfamily and has found to be a muscle-derived factor that regulates pre- and post-synaptic plasticity (Lin et al., 1993).

GDNF abides by the neurotrophic theory by being expressed in target skeletal muscle tissue, by being retrogradely transported to axonal cell bodies (Yan et al., 1995), and by providing survival support for motor neurons throughout their lifespan (Keller Peck et al., 2001). When compared to other NFs, GDNF has been shown to be up to 2500-fold more potent, being able to save nearly 100% of axotomized motor neurons and

was the only factor to prevent axotomy-induced motor neuron atrophy (Henderson et al., 1994).

Although GDNF was first identified in midbrain dopaminergic neurons (Lin et al., 1993), it has since been found in numerous sites such as skeletal muscle, Schwann cells, motor neuron axons and cell bodies (Henderson et al., 1994; Nosrat et al., 1996; Springer et al., 1994; Springer et al., 1995; Trupp et al., 1995). GDNF exerts its effects through the GDNF-GFR-α1-GPI complex, which interacts with Ret tyrosine kinase receptors that can be found localized presynaptically in the neuromuscular junction (Airaksinen and Saarma, 2002). The loss of Ret receptors decreases motor neuron survival embryonically, reduces adult motor neuron end plate numbers by half and results in a marked deficiency of maturing axon terminals within the adult muscle (Baudet et al., 2008). The overexpression of GDNF or treatment with GDNF has been shown to increase nerve terminal sprouting, slow the process of synapse elimination, and increase the number and size of end plates (Keller Peck et al., 2001; Nguyen et al., 1998; Zwick et al., 2001).

Neurotrophic factor secretion has been shown to increase following synaptic activity and may act to enhance transmission (Schinder and Poo, 2000). In the central nervous system (CNS), NFs have been shown to exhibit enhanced or reduced expression and secretion following increased or decreased synaptic activity, respectively (Lauterborn et al., 2000; Zafra et al., 1991). The amount of NF secreted has also been shown to increase in proportion to the level of synaptic activity. The presence of secreted NFs, in turn, has been shown to induce a potentiation of transmitter release from presynaptic nerve terminals (Wang and Poo, 1997). Similar findings have been shown *in vitro* where

NFs increase following chronic depolarization in culture (Lohof et al., 1993; Vianney and Spitsbergen, 2011) and *in vivo*, where NFs increase following exercise (Gómez Pinilla et al., 2002; McCullough et al., 2011; Wehrwein et al., 2002).

Exercise and GDNF independently have been shown to induce similar changes in the neuromuscular system, such as enhancing maintenance of synapses, inducing axonal sprouting (Andonian and Fahim, 1987; Keller Peck et al., 2001; Zwick et al., 2001), and increasing end plate complexity and size (Deschenes et al., 1993; Keller Peck et al., 2001). Exercise has been shown to induce synaptic activity and alter GDNF expression in skeletal muscle and spinal cord (McCullough et al., 2011; McCullough et al., 2013; Wehrwein et al., 2002). Previous studies have shown that GDNF expression is regulated in an activity dependent manner and its expression may depend on the recruitment of myofibers (McCullough et al., 2011; Wehrwein et al., 2002). For example, following low intensity exercise, an increase of GDNF expression was observed in the SOL, a predominantly slow twitch muscle, but decreased in the EDL, a predominantly fast twitch muscle (McCullough et al., 2011). This may indicate that the low speed selected for the run training (10m/min) was not of high enough intensity to recruit fast twitch myofibers and consequently decreased GDNF protein content. Swim training has been shown to demand the recruitment of fast twitch dorsiflexors more than run training, due to its higher cycling rate and to overcome the added resistance of the water medium during the recovery phase (Gruner and Altman, 1980; Roy et al., 1991). Swim training has shown similar recruitment patterns as run training in fast twitch myofibers only when the treadmill reached higher speeds of ~67m/min (Roy et al., 1991).

It is, therefore, our broad aim to examine GDNF expression and NMJ morphology in predominantly slow and fast twitch myofibers, following run and swim training. It is our hypothesis that the fast twitch myofibers will be recruited and subsequently display an increase in GDNF protein content following swim training when compared to run training (10m/min).

Methods

Subjects

Six-month old Sprague Dawley rats (Charles River, Kalamazoo, MI) were housed in rooms lighted from 7 AM-7 PM and given free access to standard rat chow and water *ad libitum*. Approval for this work was obtained from the Institutional Animal Care and Use Committee at Western Michigan University. Rats were randomly assigned to a swim group (n=6), run group (n=5) or control group (n=6). The control group was housed in individual standard living chambers and remained sedentary throughout the study.

Training Protocols

The training protocol duration was based on previous studies that observed changes in GDNF expression following two weeks of exercise (McCullough et al., 2011; McCullough et al., 2013).

Rats in the swim-train group (swim group) swam in a barrel (3/barrel) for 2 hours/day for 2 weeks. Each swim consisted of five 24-min bouts separated by 10-min rest times. The water was filled to a depth of 100 cm and maintained at 35°C, which was

large enough for each rat to swim freely as previously published (Li et al., 2012; McCullough et al., 2013).

Rats in the run train group (run group) were placed in forced running wheels (Lafayette Instruments, Lafayette, IN) for 2 hours/day for 2 weeks as previously published (McCullough et al., 2013). The wheels were set at 10m/min and each run consisted of five 24-min bouts separated by 10-min rest times to match the swim exercise.

Tissue Preparation and Storage

At the conclusion of the 2-week training period, rats from all groups were weighed and then euthanized via CO2 asphyxiation and thoracotomy. Immediately following death, the soleus (SOL) and the extensor digitorum longus (EDL) muscles were removed. The muscles from the left side of the body were used for immunohistochemical analysis. Muscles were washed with phosphate-buffered saline (PBS: 0.225 M NaCl, 0.02 M NaH2PO4, and 0.08 M Na2HPO4), frozen at resting length in isopentane and stored at -80°C. The muscles from the right side of the body were used to determine GDNF protein content using enzyme-linked immunosorbent assay (ELISA), as previously published (McCullough et al., 2011). Briefly, muscles were dipped in liquid nitrogen, smashed into fine powder, and homogenized in sample processing buffer solution (0.55 M NaCl, 0.02 M NaH2PO4, 0.08 M Na2HPO4, 2 mM EDTA, 0.1 mM benzethonium chloride, 2 mM benzamidine, 20 KIU/ml aprotinin, 0.5% BSA, and 0.05% Tween-20). Homogenates were centrifuged at 4°C for 30 min and the resultant supernatant was decanted and stored at -80°C.

Immunohistochemistry

To analyze GDNF localization and NMJ structure, the middle sections of the SOL and EDL were removed from connective tissue, embedded in optimum cutting temperature compound (Sakura Finetek, Torrance, CA, USA) and cut on a Leica microtome-cryostat. Tissues were cut horizontally at 60μm for end plate and GDNF visualization and cut transversely at 10μm for fiber type analysis. All sections were thaw mounted on HistoBond Microscope Slides (VWR, 195 International, Bridgeport, NJ, USA). To help sections adhere, slides were vacuum sealed and left overnight at 4°C. Slides were then fixed using 4% paraformaldehyde diluted in PBS at room temperature for 1h. After fixing, the tissue was incubated in buffer containing 5% normal serum from the host species for the secondary antibody. Primary antibodies were incubated on sections overnight in a humidified chamber. To identify GDNF protein, polyclonal rabbit anti-GDNF antibody was used (Santa Cruz Biotechnologies; 1:200). End plates were identified using α-bungarotoxin (1:200) directly conjugated to AlexaFluor 488®. All fiber type specific primary antibodies were purchased from Developmental Studies Hybridoma Bank, University of Iowa. The following day, slides were washed in PBS and secondary antibodies were applied at a concentration of 1:500. Secondary antibodies were conjugated to AlexaFluor 488®, AlexaFluor 568®, or AlexaFluor 647®. Slides were washed a final time followed by the application of 1:1 glycerol:PBS and a glass coverslip for microscopy.

Quantification: End Plate Size

A confocal microscope (Zeiss LSM 510) was used to visualize and collect end plate images. Once it was determined that the end plate was within the longitudinal border of the myofiber, a z-series of scans constructed a detailed picture using a C-Apochromat 63x/1.2 water correction objective (Zeiss, LLC). Fifty random endplates were captured from the EDL and SOL muscles for morphological analysis from at least three animals per group. Each end plate was measured for total area, stained area, total perimeter and stained perimeter using ImageJ software as previously described (Deschenes et al., 2006). In brief, a box was drawn around each end plate with lines touching each side of the stained area to determine total area and total perimeter. Total area included the stained regions and non-stained regions interspersed within those clusters residing inside the drawn box. Total Perimeter included the length of the box drawn around the end plate clusters that encompassed the stained and nonstained regions. Threshold was used to draw a line around only the stained area and stained perimeter of the individual end plate clusters.

Quantification: Muscle Fiber Cross Sectional Area (CSA)

Frozen transverse cross-sections (10μm) were stained against myosin heavy chain for determination of myofiber cross-sectional area (CSA) in the SOL and EDL muscles. Widefield fluorescence microscopy was used to analyze random samples of myofibers from the EDL and SOL muscles as previously published (Legerlotz et al., 2008). 125-150 myofibers were analyzed from three different animals per group to determine average myofiber CSA.

Statistics

All statistical analysis was performed using SPSS statistical software. Descriptive statistics were calculated to define means and standard errors for all variables. The results were reported as the mean ± standard error of the mean (SEM). A one-way ANOVA and Tukey post hoc test were used to assess the statistical significance among the different groups. Linear regression analysis was performed on the individual samples to evaluate the association between variables. Statistical differences were considered significant at $P<0.05$.

Results

Training Alters GDNF Protein Content

An ELISA was used in order to detect GDNF protein changes following exercise protocols in the SOL and EDL myofibers when compared to sedentary controls. An increase in GDNF protein content was observed ($P < 0.05$) following both run and swim training in the SOL muscles when compared to controls (Table 2). GDNF protein content in the EDL muscle was mostly affected following swim training. Although the increase in the swim group was not significant, it was increased by 60% ($P < 0.06$) when compared to the control group and was significantly different ($P < 0.05$) when compared to the run group.

Training Induces Changes in Weight and CSA of Muscle

The animal's body weight, relative muscle weight and myofiber CSA were measured in order to observe any changes following the two weeks of training when compared to sedentary controls.

TABLE 2.

Training Alters GDNF Protein Content

	Soleus	EDL
Control	2.5 ± 2.2	4.5 ±2.4
Run	7.6 ± 6.4*	4.0 ± 1.4
Swim	8.6 ± 4.5*	7.2 ± 3.0#

Table 2. *GDNF protein content (pg/mg tissue weight; means ± S.E.M) in the SOL and EDL muscles following exercise. GDNF content increased in the SOL following run and swim training and increased 60% from controls in the EDL swim group. Symbols denote statistical significance ($P \leq 0.05$) compared to control (*) and run group (#).*

The animals showed a decrease in their body weight following two weeks of run training ($P < 0.05$). Following swim training both the body weight and the relative muscle weight of the EDL muscle was increased when compared to the run and control groups ($P < 0.05$; Table 3).

The changes observed in the CSA of the myofibers were similar to GDNF trends in that both training protocols induced changes in the SOL muscle (Table 4). The CSA of the SOL myofibers decreased significantly following both training protocols ($P < 0.05$).

TABLE 3.

Animal Body and Muscle Weights

		Body Wt (g)	Tissue Wt (mg)	Relative Muscle Wt (mg/g)
SOL	Control	459 ± 10	298 ± 19	0.65 ± 0.04
	Run	408 ± 11*#	243 ± 31	0.65 ± 0.06
	Swim	453 ± 10	267 ± 23	0.59 ± 0.05
EDL	Control	459 ± 10	238 ± 18	0.52 ± 0.04
	Run	408 ± 11*#	247 ± 21	0.62 ± 0.03
	Swim	453 ± 10	302 ± 20*	0.67 ± 0.04*

Table 3. *Rat body weight (g) and tissue weight (mg; SOL & EDL) were measured in sedentary control animals as well as following two weeks of run and swim training. Symbols denote statistical significance ($P \leq 0.05$) compared to control (*) and run group (#). Values are means ± S.E.M.*

Neither training protocol significantly altered the CSA of the EDL myofibers when compared to controls (Table 4). Although the CSA of the EDL muscle showed an increased trend, it was only significantly different from the run group ($P < 0.05$).

TABLE 4.

Training Alters Muscle Fiber CSA

	Soleus	EDL
Control	3831 ± 180	2574 ± 98
Run	2109 ± 65*#	2352 ± 103
Swim	3243 ± 101*	2679 ± 104#

Table 4. *Myofiber cross sectional area (CSA; means ± S.E.M) in the SOL and EDL muscles following exercise. CSA decreased in the SOL following run and swim training and increased in the EDL of the swim group compared to the run group. Symbols denote statistical significance ($P \leq 0.05$) compared to control (*) and run group (#).*

Training Alters End Plate Morphology in SOL Muscle Fibers

The end plates of the SOL and EDL myofibers were measured to determine any changes in morphology following two weeks of training when compared to sedentary controls.

After two weeks of run training, no changes were observed for end plate area and size in the SOL myofibers. Run training did have an effect on measured perimeters as an increase was observed following the two weeks of training when compared to sedentary controls ($P < 0.05$; Figure 1). End plate plasticity was observed following two weeks of

swim training in the SOL muscle in all selected measurements ($P < 0.05$; Figure 1). Increases in the end plate area and total perimeter were found following two weeks of swim training when compared to the run and control groups as well as an increase in the stained perimeter when compared to the control group.

Training Alters End Plate Morphology in EDL Muscle Fibers

Two weeks of run training altered the end plate morphology of the EDL end plates by significantly reducing the stained area, total area, and total perimeter measurements when compared to sedentary controls ($P < 0.05$; Figure 2). There were no differences observed in the end plates of the swim group when compared to controls following two weeks of swim training. Although end plate measurements were not significantly different from controls, this may indicate a resistance to atrophy that was not observed following run training.

GDNF Protein Content Correlates with End Plate Total Area

Correlation statistics were ran to determine if a relationship exists between GDNF protein content and end plate measurements. GDNF protein content and end plate total area averages for all groups follow similar patterns of change (Figure 3A). A relationship exists between GDNF protein content and end plate total area for all groups ($r=0.611$, $P<0.01$, $n=30$; Figure 3B). GDNF was observed in and around the end plates as well as separate from the end plates (Figure 3C-E). This suggests that GDNF is expressed at the NMJ and in the myofibers as supported by the ELISA (Table 2). Increases in GDNF protein content and end plate area were observed in the SOL muscle following training (Figure 3C-E).

FIGURE 1.

Effects of exercise on post-synaptic endplate morphology in SOL muscle**.**

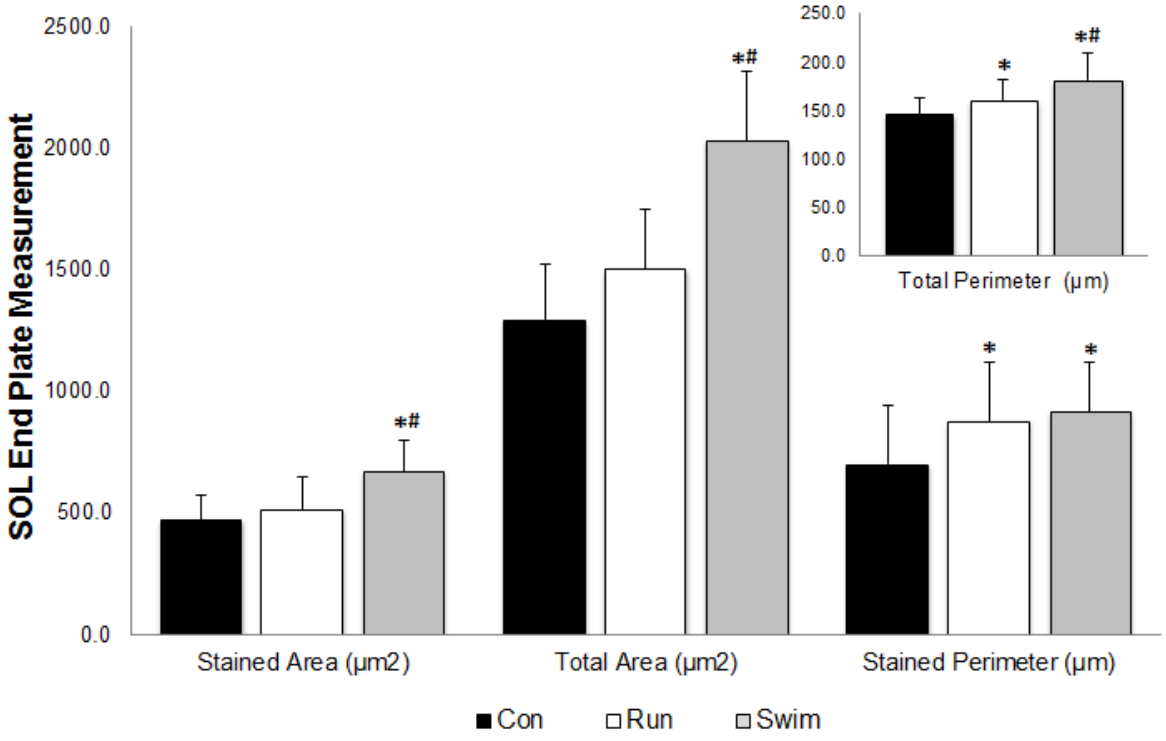

Figure 1. *Cross sections (60µm) of SOL were stained with α-bungarotoxin for visualization of motor endplates. Stained and total area (µm2) increased significantly in the swim group (gray) when compared to the run group (white) and control group (black). Stained and total perimeter (µm) increased significantly in both exercise groups when compared to control. Values are displayed as the mean ±SEM. Asterisk (*) indicates a significant (P≤0.05) difference from the control group. Number symbol (#) indicates a significant (P≤0.05) difference from the run group.*

FIGURE 2.

Effects of exercise on post-synaptic endplate morphology in EDL muscle**.**

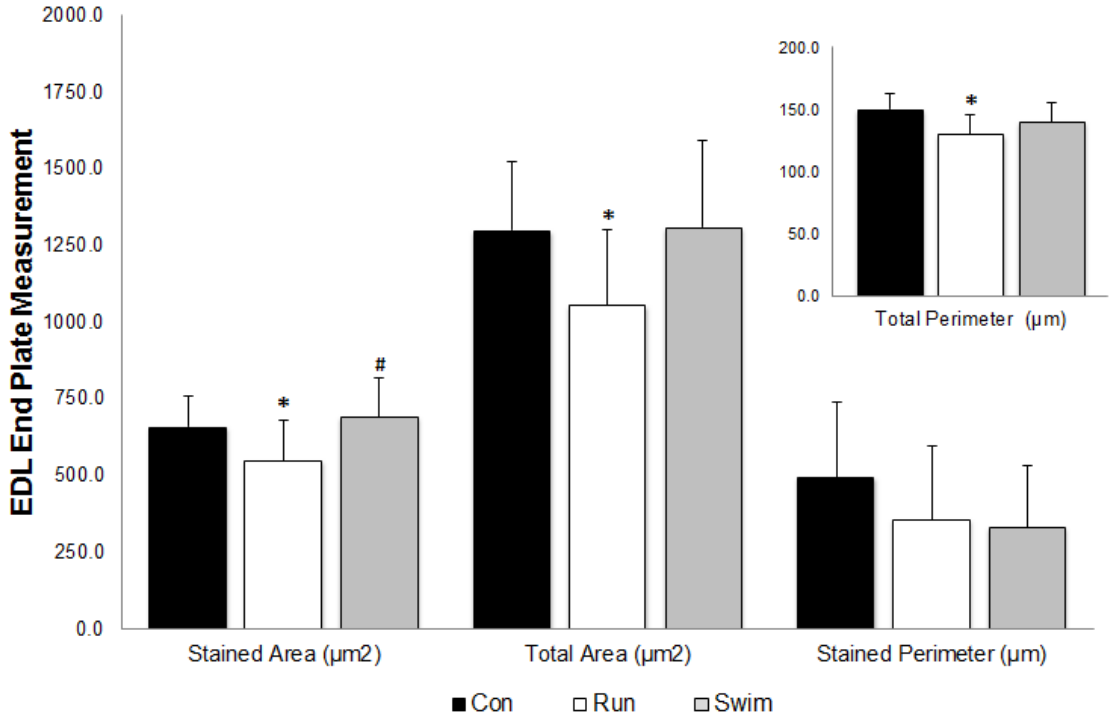

Figure 2. *Cross sections (60µm) of EDL were stained with α-bungarotoxin for visualization of motor endplates. Stained and total area (µm2) and total perimeter (µm) decreased significantly in the run group (white) when compared to the control group (black). Stained area (µm2) for the swim group (grey) increased significantly from the run group. Values are displayed as the mean ±SEM. Asterisk (*) indicates a significant (P≤0.05) difference from the control group. Number symbol (#) indicates a significant (P≤0.05) difference from the run group.*

FIGURE 3.

Correlation between GDNF levels in skeletal muscle and total end plate area**.**

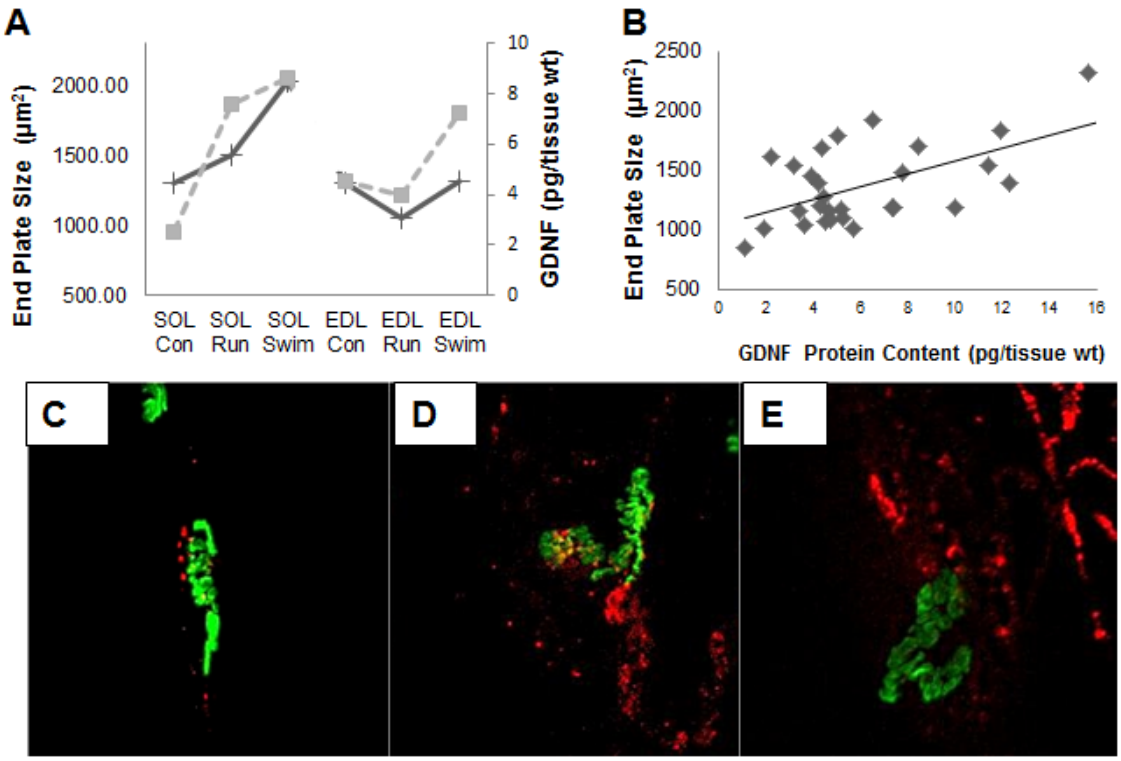

Figure 3. *End plates were visualized with α-bungarotoxin and measurements were taken using ImageJ software. Tissues were processed for GDNF protein content using ELISA and visualized by staining with anti-GDNF antibodies (blue). End plate total area (µm2) was measured by drawing a box around the entire end plate, including the stained ACh receptor clusters and the area in between. (A) Average measurements showing the overall trend for all groups, comparing GDNF protein content (dashed line) and end plate total area (solid line). (B) Levels of GDNF (pg/mg tissue wt.) were positively correlated with end plate measurements for total area (r=.611, P<0.01, n=30). (C-E) Visualization of GDNF was labeled with rabbit anti-GDNF (blue) in and around SOL end plates labeled with α-bungarotoxin (green) in control (C), run (D), and swim groups (E).*

Discussion

GDNF Expression and NMJ Plasticity in Soleus

One of the main findings of this study was the ability of the soleus muscle to increase GDNF protein content and exhibit significant plasticity at the NMJ following two weeks of run and swim training. Both training protocols were sufficient to induce an increase in GDNF protein content, further supporting an activity-dependent mechanism for neurotrophic support (McCullough et al., 2011). The observation that SOL myofiber CSA decreases with these exercise plans suggests that these muscles were recruited with these activities (Deschenes et al., 1993; Deschenes et al., 2006; Waerhaug et al., 1992). The training-induced reduction in fiber diameter is an indication of recruitment and is thought to be a positive adaptation to more readily exchange oxygen, carbon dioxide and waste products prolonging time to fatigue (Hoppeler et al., 2003).

The changes in the CSA of the SOL myofibers were most significant following the run training when compared to the swim and control groups possibly due to the differences in recruitment patterns of the two exercises selected. The two protocols place different demands on the recruitment patterns (Ariano et al., 1973) of the homogeneous slow twitch SOL muscle (Armstrong and Phelps, 1984) and the fast twitch EDL muscle (Ariano et al., 1973). The low intensity speeds selected for the run-training protocol have been shown to nearly maximize the recruitment of the slow twitch SOL myofibers while higher cycling rates, such as swimming (more steps per unit time) may decrease that activation (Roy et al., 1991).

Both training protocols were able to alter the post-synaptic apparatus of the SOL muscle, consistent with previous findings supporting exercise as a means to alter end plate morphology in fully mature rats (Deschenes et al., 1993; McCullough et al., 2011; Waerhaug et al., 1992). GDNF protein content and end plate total area in the SOL were found to be positively correlated following run training consistent with previously findings (McCullough et al., 2011). The total end plate area may increase following presynaptic changes, in order to avoid fatigue (Hill et al., 1991). This activity-dependent adaptive mechanism may increase neurotransmitter stores (Stephens and Taylor, 1972) and quantal storage and release (Dorlöchter et al., 1991). This may subsequently demand the enlargement of its counterpart, the acetylcholine (ACh) receptor field. An increase in post-synaptic ACh receptor field has been previously shown following overexpression of GDNF (Keller Peck et al., 2001; Wang et al., 2002; Zwick et al., 2001).

GDNF Expression and NMJ Plasticity in EDL

The second substantial finding in this study was the ability of swim-training to increase GDNF protein content in fast twitch myofibers by 60% compared to controls. Although this was not found to be statistically significant, it may be physiologically relevant and clinically important. These findings may provide encouragement for future studies to fine tune exercise prescription in order to recruit and support fast twitch myofibers and their innervating neurons through expression of neurotrophic factors.

The EDL was presumably recruited as evidence of hypertrophy of the EDL myofibers based on the mean absolute and relative muscle weight gain following swim training. It has been previously shown the increased cycling rates in swimming escalate

the demands for additional power via recruitment of larger fast twitch fibers (Henneman, 1981) and may be responsible for the trend towards an increase in GDNF content. Given the additional demand to meet the higher intensity swim-training and the resistance to dorsiflexion offered by the water medium, the fast twitch EDL muscle has been shown to be more heavily recruited than the slow SOL muscle during swim-training. These results suggest that with further increases in intensity, such as resistance training, a statistically significant increase in GDNF protein content in fast twitch fibers may result.

This finding may be clinically significant given that sarcopenia, the age associated decline in skeletal muscle mass and strength, is closely associated with increased frailty in the elderly, with decreased functional mobility and independence leading to a diminished quality of life, morbidity and mortality (Aniansson et al., 1984; Nevitt et al., 1989; Roubenoff, 2001). Sarcopenia appears to be fiber-type specific as large motor units that innervate fast twitch skeletal muscle present as the most vulnerable to denervation, followed by intermediate and finally the most resilient small motor units (Edström et al., 2007; Doherty, 2003; Frey et al., 2000). The denervation of large motor units causes terminal sprouting of adjacent surviving motor units to myofibers left without innervation (Rich and Lichtman, 1989). This increases the size of the remaining motor units and may cause overburdening and exhaustion of that neuron, progressively leading to further denervation and eventual disability and mortality.

Denervation has been shown to occur before myofiber atrophy and is a necessary prerequisite for muscle mass loss and fiber type conversion (Deschenes et al., 2010). Therefore, it has been suggested that by resisting the initial steps of denervation of the

large motor units, the aging process of sarcopenia may be delayed in the neuromuscular system (Deschenes et al., 2010). In addition, significant changes have been observed in fast twitch myofibers after disuse (Grimby et al., 1980) suggesting that exercise may play a key role in delaying the onset of aging in recruited fibers and in the neuromuscular system.

Both GDNF and exercise independently have been shown to protect large motor neurons from degeneration. GDNF-treated mice increased the large labeled motor neurons by 18-fold compared to β-Gal-treated mice and 3-fold more than untreated mice in a transgenic amyotrophic lateral sclerosis model (Mohajeri et al., 1999). Further studies are needed to determine if manipulation of exercise prescription can significantly increase GDNF protein content in fast twitch myofibers, leading to protection of large motor neurons.

Conclusion

These findings support that GDNF expression is activity dependent and that it may be possible to increase GDNF's protein content in slow and fast twitch myofibers following different modes and intensities of exercise. It also supports the idea that GDNF plays a role in remodeling the NMJ in slow and fast twitch myofibers. This continues to be encouraging for the use of exercise as a mechanism to increase GDNF protein content in skeletal muscle, leading to enhanced plasticity of the NMJ and enhanced neuromuscular health. With further research, guidelines can carefully inform exercise prescription that maximizes the neuromuscular benefits in both slow and fast twitch myofibers.

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CHAPTER III

GDNF CONTENT AND NMJ MORPHOLOGY ARE ALTERED IN RECRUITED MUSCLES FOLLOWING HIGH SPEED AND RESISTANCE WHEEL TRAINING

Introduction

The term sarcopenia was first coined in 1989 by Rosenberg to describe the age related decline in skeletal muscle mass (Rosenberg, 1997). It has since been shown that muscle mass alone may be a weak predictor of disability and mortality (Goodpaster et al., 2006; Newman et al., 2006; Visser et al., 2005). A relationship exists between the loss of muscle mass and loss of strength, but it is not linear due to a faster progression of the latter, leading to diminished muscle quality (muscle strength per unit of muscle mass) (Delmonico et al., 2009; Goodpaster et al., 2006). It has been suggested that muscle quality is a much better independent predictor of functional decline and mortality than loss of muscle mass alone and attention to this factor may be vital to delaying morbidity in the aging adult (Goodpaster et al., 2006; Newman et al., 2006; Visser et al., 2005).

One potential underlying cause of diminished muscle quality is the plasticity of the aging neuromuscular system, as seen with changes in motor units, peripheral nerves and the neuromuscular junction (NMJ). As with loss of muscle mass and strength, a reduction of 1% of total motor units per year can be seen beginning as early as the third decade of life and this loss increases exponentially through the sixth decade (Tomlinson & Irving, 1977). It has been predicted that between the ages of 20-90, an 80% loss of motor units (MUs) occurs with an accelerated rate of loss after the age of 60 (F. Wang, de

Pasqua, & Delwaide, 1999). Many studies on human and animal aging have shown a preferential loss of the largest and fastest conducting MUs (Ansved & Larsson, 1990; Hashizume, Kanda, & Burke, 1988; Knox, Kokmen, & Dyck, 1989; F. Wang et al., 1999) causing atrophy of type II myofibers more so than type I (Fiatarone et al., 1990; Klitgaard et al., 1990; Larsson, Ansved, Edström, Gorza, & Schiaffino, 1991). While the mean cross sectional area (CSA) of type I myofibers exceeds that of type II by 20% in the third and fourth decades of life (Brooke & Engle, 1969), by the eighth decade of life, type II CSA is less than 50% of that of type I (Tomonaga, 1977).

As a compensatory mechanism to maintain force production, collateral sprouting of nearby surviving motor axons reinnervate some, but not all type II myofibers (Pestronk, Drachman, & Griffin, 1980). This causes the surviving motor units to increase in size leading to declines in force steadiness and fine motor control (Tracy, Byrnes, & Enoka, 2004), declines in motor conduction velocities in peripheral nerve (F. Wang et al., 1999) and a decline in motor unit firing rate at effort levels relevant to activities of daily living (Ling, Conwit, Ferrucci, & Metter, 2009).

Physical activity, and specifically resistance training, has been found to be a reliable treatment to slow or reverse the declines observed in skeletal muscle through the aging process (Peterson, Rhea, Sen, & Gordon, 2010). Older sedentary individuals have been found to be twice as likely to develop severe sarcopenia compared to age-matched active individuals (Janssen, Heymsfield, & Ross, 2002). Resistance training has been found to significantly increase muscle strength, mass and functional mobility equally in both men and women (Leenders et al., 2013) and even into the 9th decade of life

(Fiatarone et al., 1990). In addition, resistance training has the ability to up-regulate protein synthesis as much as 50% in mixed myofibers in response to progressive overload (Yarasheski et al., 1999) and induce type II myofiber hypertrophy by initiating satellite cell proliferation, differentiation and fusion of new myonuclei into existing myofibers (Leenders et al., 2013). Further, only resistance training versus a low intensity run- and swim-training had the ability to maintain the mean fiber area of large type IIb fibers similar to young controls (Klitgaard et al., 1990).

One possible mechanism underlying the positive adaptations observed following resistance training in type II myofibers is the activity-induced protection of large MUs. Denervation of the motor neuron has been shown to precede, and to be a necessary prerequisite for, atrophy of the innervated myofiber (Deschenes, Roby, Eason, & Harris, 2010). In addition, it has been postulated that the net loss of myofibers in sarcopenia may be due to an impaired capacity for axonal reinnervation of denervated myofibers (Aagaard, Suetta, Caserotti, Magnusson, & Kjær, 2010). Therefore, if higher intensity exercise can recruit type II myofibers, perhaps the activity-induced protection of large MUs can prevent atrophy and loss of type II myofibers, slowing or delaying the aging process.

A likely candidate to promote the survival of motor units following exercise is a neurotrophic factor named glial cell line-derived neurotrophic factor (GDNF). GDNF is produced in target tissues of neurons, including skeletal muscle (Suzuki, Hase, Miyata, Arahata, & Akazawa, 1998) and acts in a retrograde fashion to exert nourishing effects (Yan, Matheson, & Lopez, 1995). GDNF has been shown to be the most potent

neurotrophic factor in promoting the survival of motor neurons and is the only candidate known to date to prevent motor neuron atrophy (Henderson et al., 1994; Lin, Doherty, Lile, Bektesh, & Collins, 1993). It has also been shown to regulate pre- and post-synaptic plasticity with a number of effects pertinent to sarcopenia, including causing hyperinnervation by inducing terminal sprouting, providing continuous synaptic remodeling, increasing end plate complexity and size, and protecting large MUs from degeneration (Andonian & Fahim, 1987; Keller Peck et al., 2001; Lin et al., 1993; Mohajeri, Figlewicz, & Bohn, 1999; Zwick, Teng, Mu, Springer, & Davis, 2001).

GDNF expression has been shown to increase following two weeks of training in an activity dependent manner, but only in presumably recruited muscles (McCullough et al., 2011; Wehrwein, Roskelley, & Spitsbergen, 2002). Low intensity walk training was able to increase GDNF in slow twitch myofibers, but decreased GDNF in fast twitch myofibers (McCullough et al., 2011). In addition, GDNF has been reported to increase following higher intensity exercise in fast twitch myofibers when comparing swim verse run training (Gyorkos, 2014). The difference in GDNF protein content, however, might have been due to the difference between modes of exercise rather than to intensity changes. In order to clarify that the intensity of is the factor regulating GDNF in fast twitch skeletal muscle, different intensities of exercise with the same mode of training must be compared. Therefore, it is our hypothesis that a higher intensity exercise, such as voluntary wheel training, will recruit fast twitch myofibers and subsequently alter GDNF protein content as well as induce plasticity of the NMJ.

Methods

Animals

All animal experiments were performed in accordance with the "Guide for the Care and Use of Laboratory Animals" (National Research Council) and protocols were approved by the Institutional Animal Care and Use Committee at Western Michigan University. Four-week old male Sprague-Dawley rats (n=30) were housed with 12:12-h light-dark. Rats were randomly divided into two sedentary control groups (CON4wk,n=5 and CON6wk,n=5), two involuntary running groups, one at a low velocity; 10m/min (INVOL-low, n=5) and one at a higher velocity; 20m/min (INVOL- high, n=5), and two voluntary running groups, one with resistance (VOL-R, n=5, 120g) and one without resistance (VOL-NR, n=5, 4.5g) (Table 5).

Voluntary Training Protocol

All rats were housed in a clear polycarbonate living chamber (19" x 10.5" x 8"). The chambers in the VOL-R and VOL-NR groups were attached to an activity wheel system (Lafayette Instrument, IN) that was freely accessible at all times throughout the study. Voluntary exercise was chosen as the mode of training because rats are internally motivated to run at higher intensities spontaneously and do not need noxious stimuli or external motivators such as food to induce running (Legerlotz, Elliott, Guillemin, & Smith, 2008; Sherwin, 1998). It also allows the rats to run at night when they are naturally active.

TABLE 5.

Study Design Summary

	Number	Speed ($m \cdot min^{-1}$)	Duration ($km \cdot day^{-1}$)	Length of time (wk)
CON-4wk	5	-	-	2
CON-6wk	5	-	-	2
INVOL-low	5	10	1.2	2
INVOL-high	5	20	1.5	2
VOL-R	5	30	1.5	2
VOL-NR	5	40	2.2	2

Table 5. *Summary of study design for sedentary and exercised rats. The CON4wk and CON6wk groups were housed individually and remained sedentary for two weeks. The exercise groups were also housed individually, but were exercised daily for two weeks on a voluntary (VOL-) or involuntary (INVOL-) basis.*

The activity wheels were 35 inches in diameter and attached to a braking system and a counter with an optical sensor that sent distance and velocity information to be stored on a computer. Running distance was calculated by multiplying the number of rotations of the activity wheel by the circumference of the wheel (1.1 m). The wheel activity was continuously recorded for the duration of the study.

No resistance was applied to the VOL-NR, but a load of 4.5g was necessary to overcome the inertia of the wheel. Resistance was applied to the VOL-R and calibrated

daily to ensure 120g of load was added to each wheel continuously for the length of the study. Calibration was accomplished by hanging 120g of known weight on the wheel bar furthest from the axis and precisely tuning the braking system until the wheel could not be displaced.

Involuntary Training Protocol

The involuntary running groups consisted of a low velocity running group (INVOL-low; 10 $m \cdot min^{-1}$, n=5) and a higher velocity running group (INVOL-high; 20 $m \cdot min^{-1}$, n=5) both being forced to exercise in motorized wheels. The INVOL-low group ran for five 24 min bouts separated by 5 min of rest time as previously published (Gyorkos, 2014; McCullough et al., 2011), while the INVOL-high group ran for five 15 min bouts separated by 5 min of rest times.

Tissue Collection and Processing

At the completion of the two weeks of training, the rats were weighed and euthanized by CO_2 asphyxiation and thoracotomy. Immediately, the soleus (SOL), plantaris (PLA), and extensor digitorum longus (EDL) were excised from the hindlimbs and weighed. These muscles were chosen in this study to determine any potential differences between predominately slow (SOL) and fast twitch (EDL & PLA) myofiber phenotypes (Ariano, Edgerton, & Armstrong, 1973; Armstrong & Phelps, 1984) following exercise.

The muscles on the left side of the body were frozen at normal length in isopentane on dry ice and stored at -80°C and used for immunohistochemistry. The

muscles on the right side of the body were further processed and used for detection of GDNF protein content via enzyme-linked immunosorbent assay (ELISA), as previously published (McCullough et al., 2011). Each muscle was dipped in liquid nitrogen, smashed into fine powder and homogenized with sample processing buffer (0.55 M NaCl, 0.02 M NaH_2PO_4, 0.08 M Na_2HPO_4, 2 mM EDTA, 0.1 mM benzethonium chloride, 2 mM benzamidine, 20 KIU/ml aprotinin, 0.5% BSA, and 0.05% Tween-20). Homogenates were centrifuged and supernatants were collected and stored at -80°C.

Immunohistochemistry

The midbelly portions of the muscles were embedded in optimum cutting temperature compound (Sakura Finetek, Torrance, CA, USA) and sectioned using a Leica microtome-cryostat. Sections were cut at 60µm for visualization of GDNF localization and NMJ structure and cut at 10µm for fiber CSA quantification.

All sections were thaw mounted on HistoBond Microscope Slides (VWR, 195 International, Bridgeport, NJ, USA), vacuum sealed and stored overnight at 4°C.

Analysis of GDNF Localization and NMJ Structure

Rat muscle serial longitudinal sections (60µm) were fixed in 4% paraformaldehyde at room temperature for 1 hour, washed in PBS (3X5 min) and incubated in buffer containing 10% donkey serum, 4% BSA, 0.5% Triton X-100 in PBS for 30 minutes in a humidified chamber at room temperature. Slides were then incubated with a primary polyclonal rabbit α-GDNF (Santa Cruz Biotechnologies; 1:200) overnight at 4°C. Slides were washed in PBS the next day and incubated for 2 hours at room

temperature in a secondary antibody cocktail including donkey α-rabbit conjugated to Alexa Fluor 647 (Molecular Probes, 1:1000) to visualize GDNF and α-bungarotoxin directly conjugated to AlexaFluor 488 (Molecular Probes, 1:1000) for end plate visualization. All antibodies were diluted in PBS containing 1% bovine serum albumin and 0.1% triton X-100. Slides were washed the next day in PBS and mounted in PBS: Glycerol (1:1) for microscopy. Images were viewed with a Zeiss Axiovert 100M confocal microscope (Zeiss LSM 510).

The sections stained with α-bungarotoxin were used for quantifying end plate measurements. Fifty random end plates were captured for each muscle (SOL, PLA and EDL) in the study using the confocal microscope with a C-Apochromat 63x/1.2 water correction objective. Each end plate was visually analyzed to make certain that it was within the longitudinal border of the myofiber before being scanned and stored. Each captured end plate was analyzed using Image J software as previously described (Deschenes, Tenny, & Wilson, 2006). Briefly, a box was drawn around each end plate with the lines of the box touching each side of the stained area. The area within the box represented the total area and total perimeter measurements of the end plate. This area included the stained as well as the non-stained area interspersed between the end plate clusters. The image was then thresholded to identify the alpha-bungarotoxin staining and represented the stained area and stained perimeter. Dispersion was measured by dividing the stained area by the total area of the end plate; therefore a lower percentage would indicate a more dispersed synapse.

Determination: Myofiber Cross Sectional Area (CSA)

The myofiber cross sectional area of the muscle fibers were determined as previously published (Legerlotz et al., 2008). 125-150 random muscle fibers were captured for each muscle (SOL, PLA and EDL) in the study using widefield fluorescence microscopy. Each muscle fiber was encircled and analyzed using Image J software.

Statistics

All statistical analysis was performed using SPSS statistical software. Descriptive statistics were calculated to define means and standard errors for all variables. The results were reported as the mean ± standard error of the mean (SEM). Data were analyzed using a one-way analysis of variance (ANOVA) and Tukey's post hoc comparison to test for statistical significant differences between groups. Linear regression analysis was performed on the individual samples to evaluate the association between variables. Differences were considered statistically significant at $P<0.05$.

Results

Training

On a daily average, the VOL-NR group ran ~33% faster (~40 $m \cdot min^{-1}$ verse ~30 $m \cdot min^{-1}$; $P<0.05$) and ~46% longer (~2.2 $km \cdot day^{-1}$ verse ~1.5 $km \cdot day^{-1}$; $P<0.05$) than the VOL-R group. The amount of work averaged over the two weeks of training were similar between groups when factoring in the 120g of resistance added to the VOL-R group using a previously published equation (W=force (N) X distance (ms)/body wt (kg)) (Ishihara, Roy, Ohira, Ibata, & Edgerton, 1998). The involuntary running protocols

matched closely in running distance for INVOL-low ($10m \cdot min^{-1}$) and INVOL-high groups ($20m \cdot min^{-1)}$ as they ran for $1.2km \cdot day^{-1}$ and $1.5\ km \cdot day^{-1}$, respectively.

Animal Body Weight and Muscle Weights

Body weight decreased ($P < 0.05$) following all of the exercise training protocols with the exception of VOL-R training (Table 6). Although the absolute tissue weight was significantly altered in all three muscle groups (SOL, PLA, EDL) depending on the exercise, only the relative weight of the PLA muscle was increased ($P < 0.05$) following voluntary exercise (VOL-R and VOL-NR). The relative weight of the SOL and EDL muscles remained unaltered following exercise protocols when compared to sedentary controls.

Effects of Exercise on PLA: GDNF

CSA was measured as an indicator of muscle recruitment where slow and fast twitch myofibers have been shown to respond differently by decreasing and increasing CSA, respectively (Deschenes et al., 1993; Deschenes et al., 2010; Waerhaug, Dahl, & Kardel, 1992). The CSA of the PLA myofibers increased significantly following both VOL-R and VOL-NR exercise when compared to age-matched sedentary controls (Figure 4; bar graph). In those recruited muscles, GDNF protein content increased 174% ($P < 0.05$) following VOL-R exercise and 161% ($P < 0.05$) following VOL-NR exercise when compared to age matched sedentary controls (Figure 4; line graph). There were no significant differences in PLA GDNF protein content or CSA between VOL-R and VOL-NR training groups, or following involuntary exercise compared to age-matched sedentary controls.

TABLE 6.

Animal Body and Muscle Weights (Values are means ± S.E.M.)

		Body Wt (g)	Tissue Wt (mg)	Relative Muscle Wt (mg/g)
SOL	Control	264 ± 12	149 ± 19	0.56 ± 0.04
	INVOL-low	217± 14*	113 ± 32*	0.52 ± 0.05
	INVOL-high	205 ± 15*	95 ± 28*	0.46 ± 0.07
	VOL-R	272 ± 14	133 ± 23	0.49 ± 0.05
	VOL-NR	230 ± 15*	128 ± 20*	0.55 ± 0.06
PLA	Control	264 ± 12	234± 21	0.88± 0.03
	INVOL-low	217± 14*	258 ± 19	1.10± 0.04
	INVOL-high	205 ± 15*	240 ± 28	1.17± 0.06
	VOL-R	272± 14	351± 18*	1.29± 0.03*
	VOL-NR	230± 15*	293± 26*	1.27± 0.06*
EDL	Control	264 ± 12	142 ± 18	0.54 ± 0.06
	INVOL-low	217± 14*	119 ± 16*	0.57 ± 0.07
	INVOL-high	205 ± 15*	125 ± 18*	0.57 ± 0.05
	VOL-R	272± 14	158 ± 19	0.58 ± 0.04
	VOL-NR	230± 15*	131 ± 26	0.57 ± 0.06

Table 6. *Rat body weight (g), absolute (mg) and relative tissue weights (mg/g) were measured in sedentary control animals and following training protocols. The relative muscle weight of the PLA increased following voluntary exercise (VOL-R and VOL-NR). Symbols denote statistical significance ($P \leq 0.05$) compared to control (*).*

FIGURE 4.

GDNF protein content increases in recruited muscles**.**

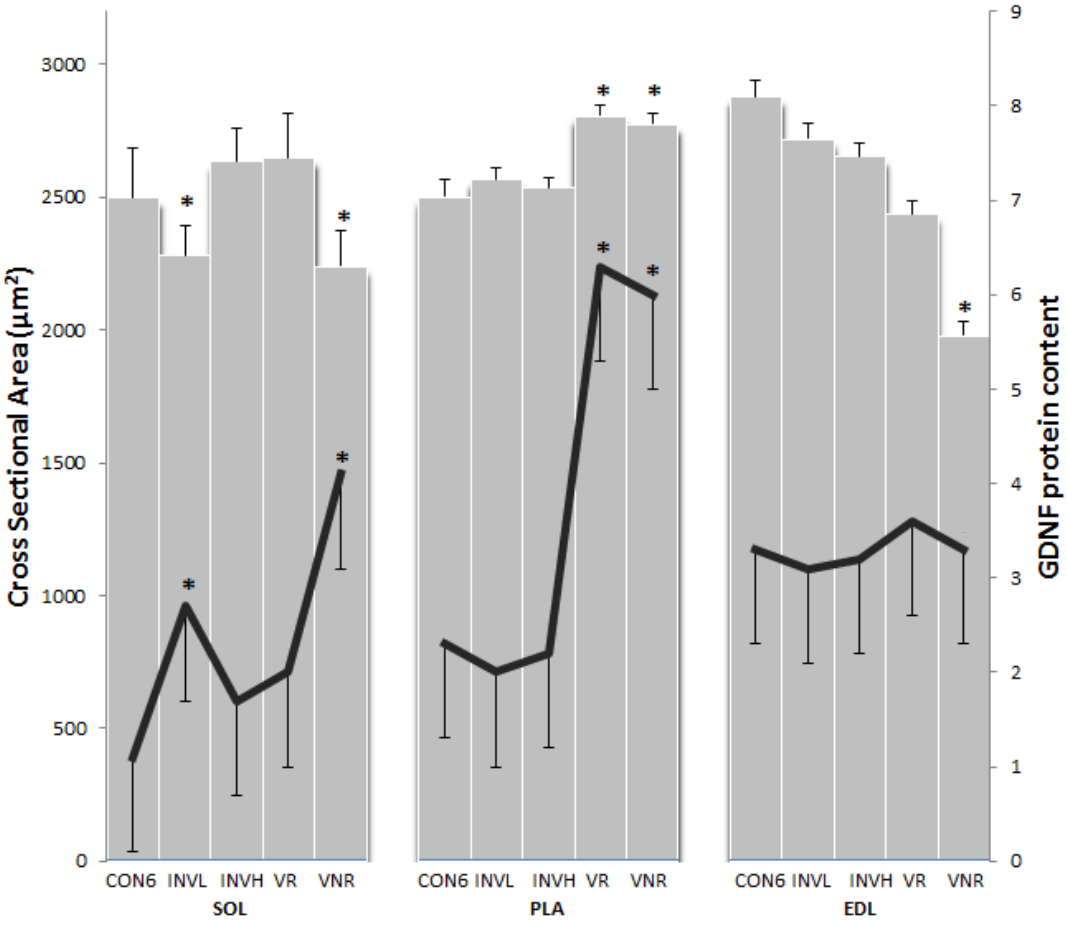

Figure 4. *GDNF protein content increases in recruited muscles. Quantification of GDNF was accomplished via ELISA. Cross sectional area (CSA; bar graph) was measured in 125-150 random SOL, EDL, and PLA fibers that were captured by widefield microscopy and analyzed using ImageJ software. GDNF protein content (pg/mg/tissue weight; line graph), determined by ELISA, was significantly increased in all recruited fibers of the SOL and PLA muscles, evidence by CSA decreasing and increasing, respectively. Increases in GDNF protein content were observed in SOL*

muscle following INVOL-low and VOL-NR training. Increases in GDNF were observed in PLA muscle following both voluntary training protocols. The EDL did not appear to be recruited following any training and no changes in GDNF were observed. Values are displayed as the mean ±SEM. Asterisk (*) indicates a significant (P≤0.05) difference from the age-matched control group. Key: CON6 (CON6wk), INVL (INVOL-low), INVH (INVOL-high), VR (VOL-R), and VNR (VOL-NR).

Effects of Exercise on PLA: NMJ Plasticity

Immunohistochemistry revealed that all of the measurements quantified for end plates in the PLA myofibers were altered following voluntary exercise. Total area, stained area, total perimeter and stained perimeter were all increased in the VOL-R and VOL-NR groups compared to age-matched controls ($P < 0.05$; Figure 5). The end plate stained area increased 123% ($P < 0.05$) and 72% ($P < 0.05$) following VOL-R and VOL-NR training, respectively, when compared to age-matched controls. Voluntary training groups showed increased end plate measurements compared to age-matched controls. The end plates increased even further following VOL-R training when compared to VOL-NR training. A positive relationship exists between PLA GDNF protein content and end plate area ($r=0.880$, $P<0.01$, $n=15$) (Figure 6 and 7).VOL-R training also resulted in more dispersed synapses when compared to VOL-NR training and age-matched control groups ($P < 0.05$; Figure 5 inset). There were no significant differences observed in end plate measurements following involuntary exercise compared to age-matched sedentary controls.

Effects of Exercise on SOL: GDNF

The CSA of the SOL myofibers decreased following INVOL-low and VOL-NR training when compared to age-matched sedentary controls (Figure 4; bar graph). SOL GDNF protein content increased 145% ($P < 0.05$) and 272% ($P < 0.05$) following INVOL-low and VOL-NR training, respectively, when compared to age-matched controls (Figure 4; line graph). There were no significant differences observed in measurements of fiber CSA and GDNF following INVOL-high and VOL-R training compared to age-matched sedentary controls.

Effects of Exercise on SOL: NMJ Plasticity

The total and stained area and total perimeter of the SOL end plates increased following INVOL-low and VOL-NR training (Figure 8). Stained area of SOL end plates increased 89% ($P < 0.05$) and 100% ($P < 0.05$) following INVOL-low and VOL-NR training, respectively, when compared to age-matched sedentary controls. There were no significant differences in dispersion of the SOL end plates. A relationship exists between GDNF protein content and end plate area ($r=.880$, $p<0.01$, $n=15$) (Figure 6 and 7). There were no significant differences observed in end plate measurements following INVOL-high and VOL-R training compared to age-matched sedentary controls.

FIGURE 5.

Effects of exercise on post-synaptic end plate morphology in PLA muscle**.**

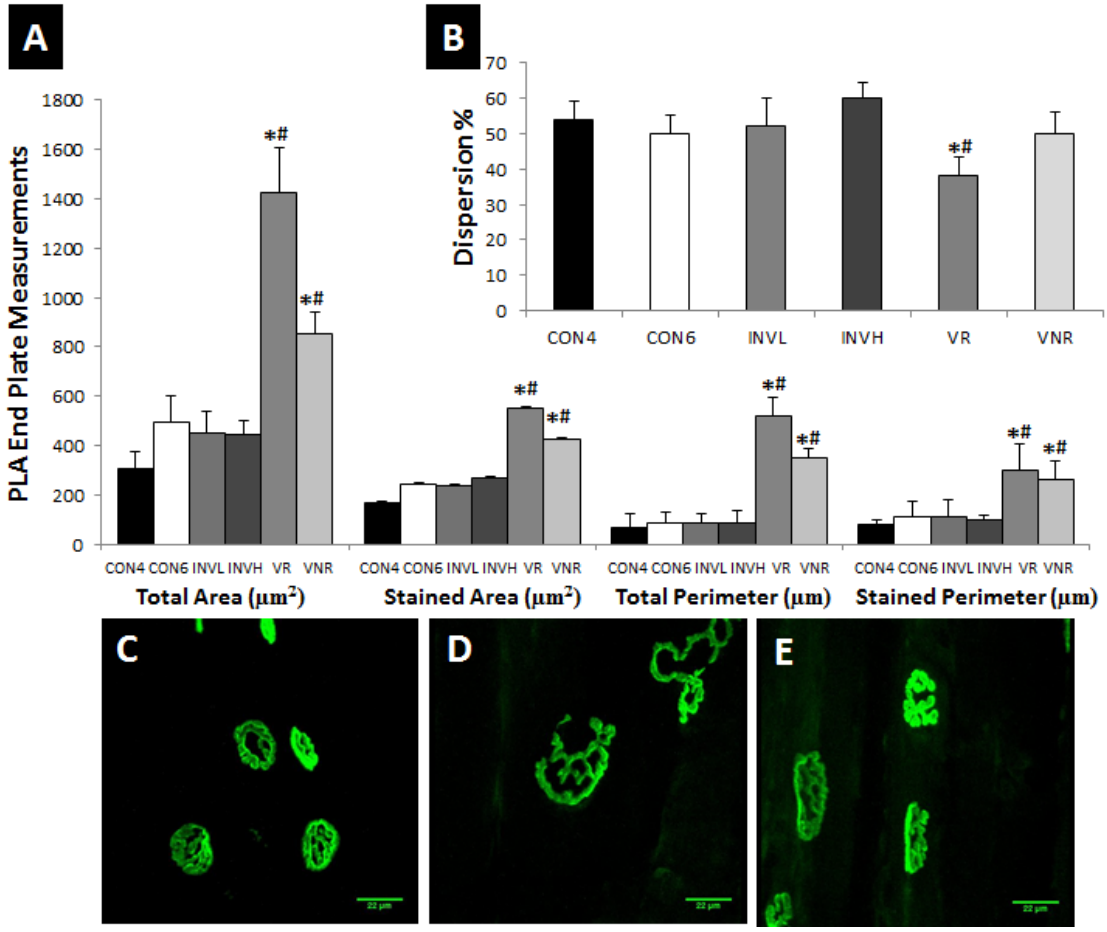

***Figure* 5.** *Effects of exercise on post-synaptic end plate morphology in PLA muscle. Cross sections (60µm) of SOL muscle fibers were stained with α-bungarotoxin for quantification and visualization. Total and stained area (µm^2) and total and stained perimeter (µm) were altered following VOL-R and VOL-NR training when compared to age-matched sedentary controls (A). In addition, VOL-R training resulted in more dispersed synapses compared to the 6wk control group (B). End plates are shown for the 6wk CON (C), VOL-R (D) and VOL-NR (E) groups. Note the dispersion observed*

following VOL-R training (D). Values are displayed as the mean ±SEM. Asterisk (*) indicates a significant (P≤0.05) difference from the age-matched sedentary control group. Number symbol (#) indicates a significant (P≤0.05) difference from the 4wk control group. Key: CON4 (CON4wk), CON6 (CON6wk), INVL (INVOL-low), INVH (INVOL-high), VR (VOL-R), and VNR (VOL-NR).

FIGURE 6.

Correlation between GDNF levels in skeletal muscle and end plate stained area**.**

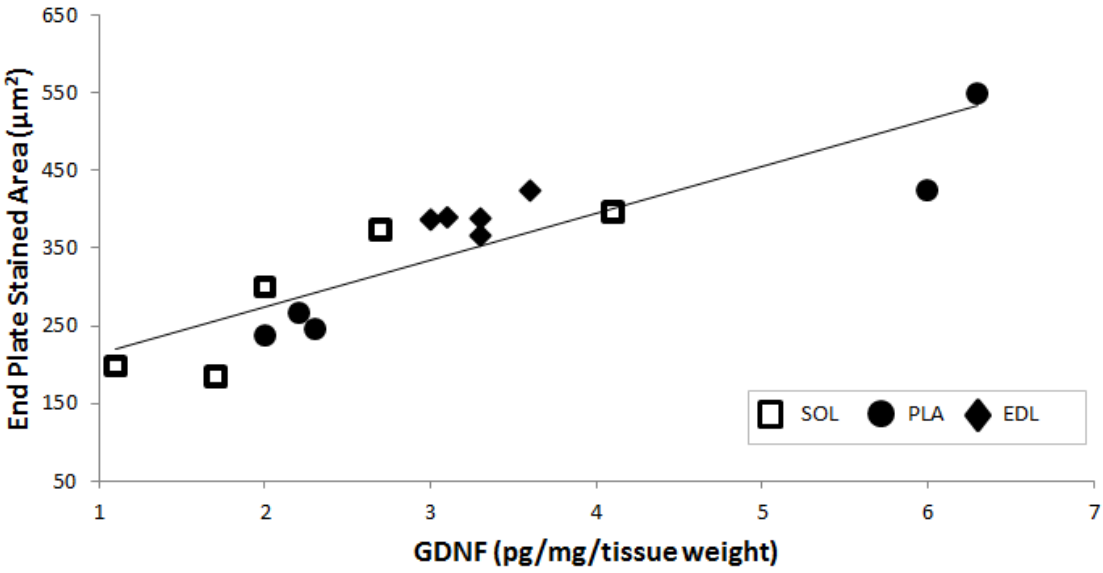

Figure 6. *Correlation between GDNF levels in skeletal muscle and end plate stained area. A relationship exists between GDNF protein content (pg/mg/tissue weight) and end plate stained area (µm²) (r=0.880, P<0.01). Each point represents one of the three muscles (SOL, PLA, EDL) in one of five groups (CON6wk, INVOL-low, INVOL-high, VOL-R, and VOL-NR), giving fifteen data points (n=15).*

FIGURE 7.

Relationship between GDNF protein content and end plate stained area.

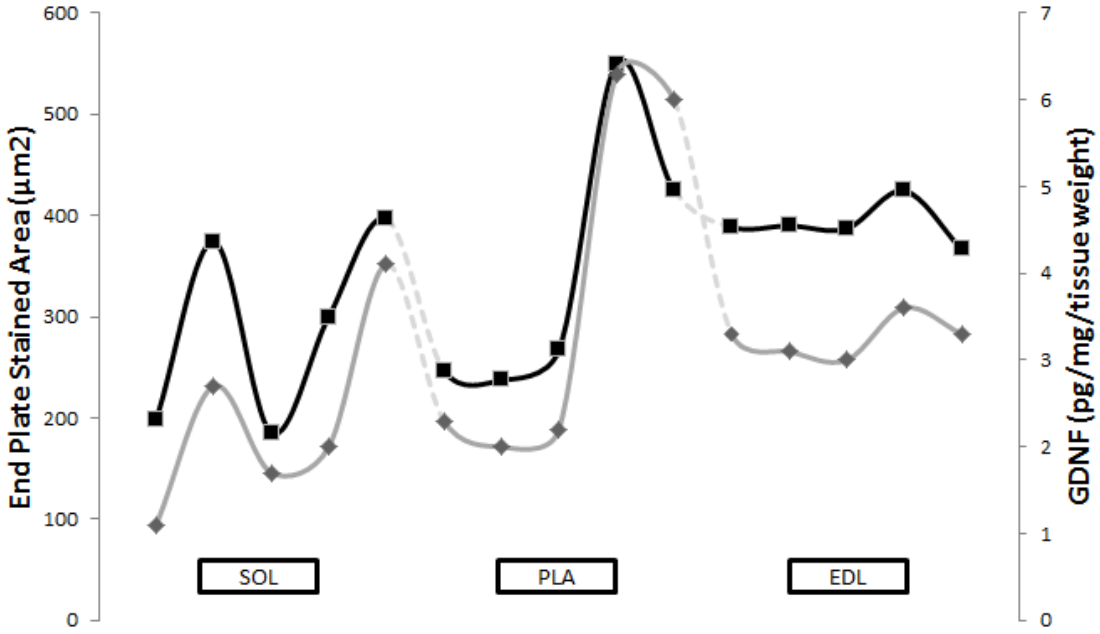

Figure 7. *Relationship between GDNF protein content and end plate stained area. This graph displays the close relationship between GDNF and end plate area in the SOL, PLA, and EDL muscles.*

FIGURE 8.

Effects of exercise on post-synaptic end plate morphology in SOL muscle**.**

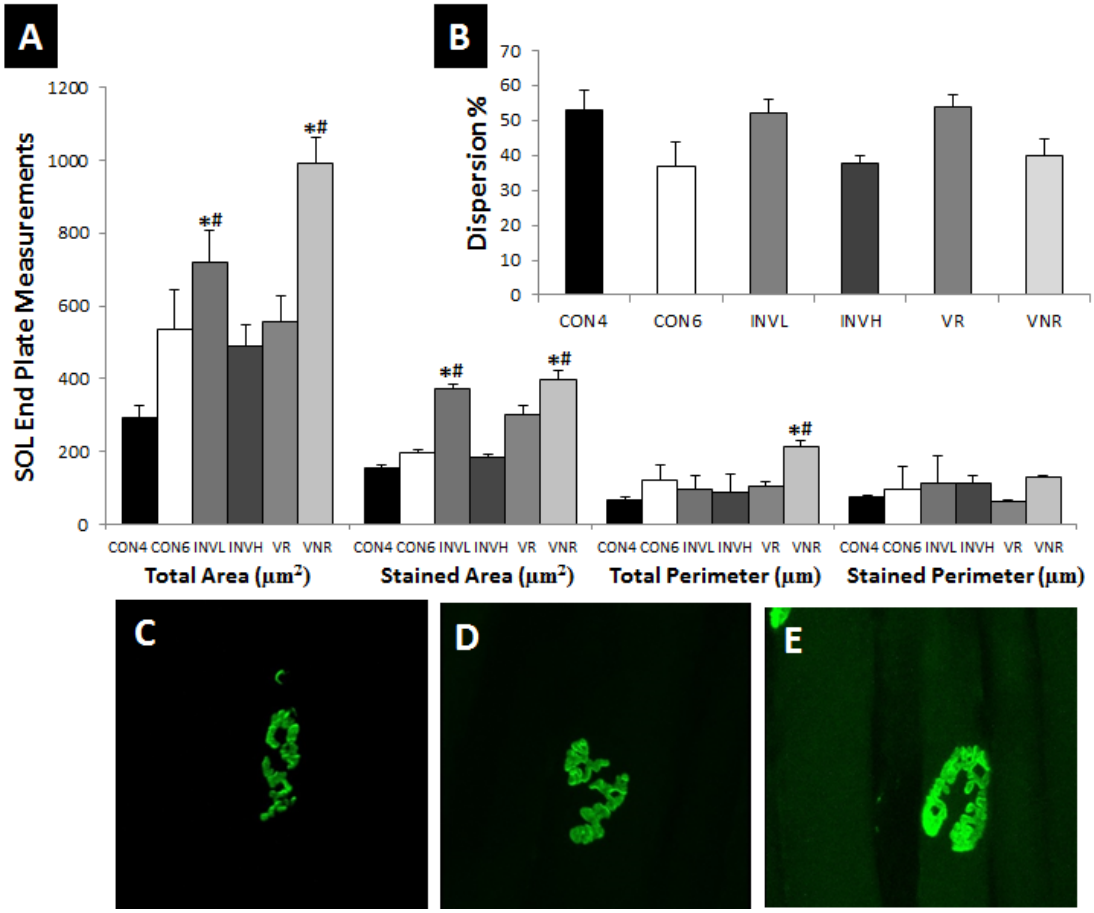

Figure 8. *Effects of exercise on post-synaptic end plate morphology in SOL muscle. Cross sections (60µm) of SOL muscle fibers were stained with α-bungarotoxin for quantification and visualization. Total and stained area (µm^2) were altered following INVOL-low and VOL-NR training when compared to age-matched sedentary controls (A). In addition, total perimeter was increased following VOL-NR training. No significant differences in dispersion were observed (B). End plates are shown for the 6wk CON (C), INVOL-low (D) and VOL-NR (E) groups. Values are displayed as the*

mean ±SEM. Asterisk (*) indicates a significant (P≤0.05) difference from the age-matched sedentary control group. Number symbol (#) indicates a significant (P≤0.05) difference from the 4wk control group. Key: CON4 (CON4wk), CON6 (CON6wk), INVL (INVOL-low), INVH (INVOL-high), VR (VOL-R), and VNR (VOL-NR).

Effects of Exercise on EDL: GDNF

The CSA of the EDL myofibers decreased following VOL-NR training compared to age-matched sedentary controls (Figure 4; bar graph). There were no other significant differences observed in measurements of fiber CSA and GDNF (Figure 4; line graph) compared to age-matched sedentary controls.

Effects of Exercise on EDL: NMJ Plasticity

There were no significant differences observed among end plate measurements when compared to age-matched sedentary controls. There were no differences in the dispersion of the EDL end plates when compared to controls. A relationship exists between GDNF protein content and end plate area ($r=.880$, $p<0.01$, $n=15$) (Figure 6 and 7).

Discussion

This is the first study to our knowledge that has shown an increase in GDNF protein content in fast twitch myofibers. GDNF levels in fast twitch myofibers have been found to significantly decrease following short term exercise at low velocities (10 $m \cdot min^{-1}$) (McCullough et al., 2011). We were able to show that by increasing both the

velocity (30-40 $m \cdot day^{-1}$) and the resistance (120g) of the training, the fast twitch myofibers of the PLA muscle were recruited and subsequently increased GDNF content.

Rats will spontaneously and naturally run at speeds close to their maximal aerobic capacity in short bouts mimicking sprint training (Rodnick, Reaven, Haskell, Sims, & Mondon, 1989; Shepherd & Gollnick, 1976). It has been shown that the high intensity nature of voluntary exercise can induce increases in enzymatic activity in fast twitch myofibers and that these changes are unlikely caused by hormonal changes, but may be a result of different patterns of muscle recruitment following higher intensity training (Bagby, Johnson, Bennett, & Shepherd, 1986; Rodnick et al., 1989). Speeds as low as 25-40m/min^{-1} have been found to significantly deplete glycogen, increase lactate accumulation and increase cytochrome *c* concentration in fast twitch myofibers (Armstrong, Saubert,C.W.,,IV, Sembrowich, Shepherd, & Gollnick, 1974; Baldwin, Campbell, & Cooke, 1977; Dudley, Abraham, & Terjung, 1982) indicating recruitment of those myofibers. In the present study, rats were able to reach speeds between approximately 30-40m/min^{-1} on average through voluntary exercise, which reach the intensity threshold for recruitment (Armstrong et al., 1974; Baldwin et al., 1977; Dudley et al., 1982).

Addition of a load of 120g of resistance within the first two weeks of the study was enough to induce hypertrophy in the PLA muscle as previously reported (Ishihara et al., 1998; Legerlotz et al., 2008). An increase in the CSA of the plantaris muscle has been shown to follow an increase in load (Ishihara et al., 1998; Legerlotz et al., 2008)

or an increase in duration (Kariya, Yamauchi, Kobayashi, Narusawa, & Nakahara, 2004) of the training period. The voluntary exercise reached an intensity that was high enough to recruit the fast twitch myofibers in both training groups and may have contributed to an increase in GDNF protein content in the PLA muscle. This is clinically relevant as this indicates that higher intensity training may be able to support the innervating neurons of those vulnerable fast twitch myofibers through increased expression of neurotrophic factors.

We were also able to show that the training altered the end plate morphology of the recruited muscles, consistent with previous findings that exercise can induce changes to the post-synaptic apparatus in rats (Deschenes et al., 1993; McCullough et al., 2011; Waerhaug et al., 1992). Deschenes et al., observed increases in the dispersion of end plates of fast twitch myofibers following higher intensity training (Deschenes et al., 1993). Our study showed similar results as the PLA end plates were more dispersed following VOL-R training, but not following VOL-NR training. It has been postulated that this pattern of dispersion following higher intensity exercise may be the result of longer and more complex arborization of the pre-synaptic terminal, compared to the shorter primary branches observed following lower intensity exercise (Deschenes et al., 1993). These results suggest that the intensity of exercise alters the NMJ of slow and fast twitch myofibers in different ways.

GDNF, in the absence of exercise, has also been found to cause continuous synaptic remodeling of the NMJ by inducing hyperinnervation, increasing end plate size and complexity, and maintaining the post-synaptic apparatus (Keller Peck et al.,

2001; C. Wang et al., 2002; Zwick et al., 2001). The results of this study are consistent with those findings as GDNF protein content was positively correlated with end plate size. It has been postulated that pre-synaptic changes, such as terminal sprouting, increased neurotransmission stores, and quantal storage and release may warrant changes in the post-synaptic apparatus in order to enhance communication and efficiency at the NMJ (Dorlöchter, Irintchev, Brinkers, & Wernig, 1991; Hill, Robbins, & Fang, 1991; Keller Peck et al., 2001; Stephens & Taylor, 1972; Zwick et al., 2001).

It is noteworthy that the muscles that were not actively recruited; PLA during involuntary training, SOL during INVOL-high and VOL-R training, and EDL following all training, with the exception of VOL-NR, displayed no changes in NMJ morphology and GDNF levels remained similar to those in sedentary controls. These findings provide further support that the regulation of GDNF is activity dependent, as its expression relies on the recruitment of the myofibers during physical activity (McCullough et al., 2011; Wehrwein et al., 2002).

Physical activity levels and intensity decline with aging, with an associated decline in skeletal muscle mass and strength (Janssen et al., 2002; Rosenberg, 1997). Those losses in mass and strength result in significant public health problems with associated increases in falls, fractures and frailty, declines in functional mobility and independence, leading to a diminished quality of life, morbidity and mortality (Aniansson, Zetterberg, Hedberg, & Henriksson, 1984; Janssen et al., 2002; Nevitt, Cummings, Kidd, & Black, 1989; Roubenoff, 2001). It has been postulated that there is no other decline in aging that is as dramatic or as significant as that of lean body

mass (Rosenberg, 1997). The decline of 3-8% of muscle mass per decade begins as early as thirty years of age with approximately 45% of the U.S. population sarcopenic and 20% functionally disabled as they approach 65 years of age (Manton & Gu, 2001). The population over 60 years old is predicted to triple in the next fifty years and with it comes diminished quality of life and disability, leading to economic costs for government-reimbursed healthcare, unless underlying mechanisms are identified and preventative measures are taken (Janssen et al., 2002).

Being physically active, on the other hand, has been shown to compress morbidity and have a 'squaring off' effect on the disability and mortality curves so that people can live longer productive lives and die after a limited period of disability (Fries, 2002). In this context, exercise has been compared to a non-pharmacological fountain of youth (Joyner & Barnes, 2013). In addition, physical fitness has been shown to be the number one independent risk factor for both all-cause and cardiovascular morbidity (Kodama S, Saito K, Tanaka S,et al, 2009).

GDNF is a muscle-derived, activity-dependent factor that may play a critical role in the positive outcomes of exercise. GDNF may act to promote the survival of motor units and therefore play a pertinent role in delaying the onset of aging of the neuromuscular system. The effects of exercise as a regulatory factor of GDNF expression deserves continued research in order to inform exercise prescription to induce changes in those myofibers that are most susceptible to degeneration, preserving nerve and muscle function and protecting the neuromuscular system.

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CHAPTER IV

DISCUSSION AND SUMMARY

Discussion

The health of the neuromuscular system is the most noticeably stricken system associated with the inescapable aging process with muscle mass decreasing by ~40% between the ages of 20-60 years and strength beginning to decline in the sixth decade of life and proceeding at a rate of ~1.3% per year thereafter (Deschenes, Roby, & Glass, 2011; Kanda & Hashizume, 1998; Koopman & van Loon, 2009; Ling, Conwit, Ferrucci, & Metter, 2009; Roos, Rice, & Vandervoort, 1997; Vandervoort, 2002). The early prescription of exercise has been shown to delay the onset of the aging process, acting to enhance one's quality of life throughout more of their life. A "reversal" of the aging signature in skeletal muscle in regards to strength, function and muscle mass has been observed following high intensity exercise (Melov, 2007).

NMJ Remodeling

There are many factors that can remodel the structural, functional, and physiological characteristics of the NMJ. Exercise has been shown to be a potent promoter of plasticity in the neuromuscular system. Specifically, resistance exercise has been shown to enhance NMJ size, which increases acetylcholine receptor number, quantal content release and endplate potential amplitude (Andonian & Fahim, 1987; Desaulniers, Lavoie, & Gardiner, 1998; Deschenes et al., 2000). Our results are consistent with these findings as the post synaptic end plate increases in size and complexity following resistance training (Chapter 3). This enhanced size and

dispersion of the end plates occurs to avoid disturbing the relationship between pre- and post-synaptic components in which the neurotransmitter release and their respective binding sites remain in close proximity (Deschenes et al., 2000; Sieck & Regnier, 2001). As the pre-synaptic component grown by increasing terminal branching, which is robust in fast twitch myofibers, the reciprocal end plate increases in size to maintain and optimize the relationship. Under normal untrained conditions the acetylcholine receptors are packed together as densely as possible within a single end plate (Desaulniers et al., 1998; Deschenes et al., 1993). The dispersion, therefore allows for additional receptors to be added to the post-synaptic field to sensitize binding sites as a response to repeated intense neural stimulation (resistance training). This end plate plasticity increases fatigue resistance and the efficacy of neurotransmission.

In addition, prolonged endurance activity, has been shown to increase protein synthesis and increase the protein transport efficiency. Trained motor neurons not only can transport larger amounts of proteins, but can increase the speed of transport in a retrograde and anterograde fashion (Jasmin, Lavoie, & Gardiner, 1988; Kang, Lavoie, & Gardiner, 1995). These findings are substantial for trophic factors that are reliant on anterograde and retrograde transport for neuronal homeostasis and cell function. One potential explanation for the loss of large motor units through aging and neurodegenerative diseases is the observed slowing of transport of trophic factors in the aged animals (Cross, Flexman, Anzai, Maravilla, & Minoshima, 2008; Stromska, Ochs, & Muller, 1981).

Interestingly, there is evidence that the age-related remodeling of the NMJ occurs in a retrograde direction, in that the denervation/re-innervation process characterizing the effects of aging and disease, begins at the NMJ and progresses towards the axonal cell body (Ansved & Larsson, 1990; Dupuis et al., 2009). It is not surprising therefore that the loss of motor neuron count at the cell body is relatively modest in comparison to denervation at the NMJ until very old age (Chai, Vukovic, Dunlop, Grounds, & Shavlakadze, 2011). It has also been observed that the denervation process of the motor neuron retracting from the muscle target is a necessary prerequisite for myofiber loss associated with diminished muscle quality (Deschenes, Roby, Eason, & Harris, 2010) with large motor units being the first to show degenerative changes detected at the earliest stages of aging (Andonian & Fahim, 1987; Cardasis & LaFontaine, 1987; Deschenes et al., 2010) . Taken together, finding a mechanism to upregulate expression of trophic factors at the NMJ and that are responsible for the continual remodeling of the NMJ, particularly in large motor units, are essential to delaying the aging and disease neurodegenerative process.

GDNF Regulation

GDNF can be upregulated in fast twitch skeletal muscle following two weeks of voluntary exercise (Chapter 3). Voluntary exercise, when compared to involuntary, was the only protocol capable of inducing significant changes in GDNF protein content in the fast twitch myofibers. Rats will run at short bursts at high velocities when given the opportunity through voluntary exercise and as such was the only protocol in which the rats ran at high speeds (>30m.min). By increasing the intensity

of exercise, through speed and resistance, the animals were able to recruit the fast twitch PLA muscle and subsequently increase GDNF protein content. This is the first time that the lab was able to show an increase in GDNF protein content in fast twitch muscle (Gyorkos & Spitsbergen, 2014). There was also a relationship between GDNF protein content and the size of the end plates (Chapter 2 & 3). This relationship is likely due to a response by the end plates to match pre-synaptic plasticity as previously described above. Our results are consistent with findings that GDNF, when overexpressed or injected, has been able to induce hyperinnervation, increased end plate size and increased pre-synaptic terminal sprouting (Henderson et al., 1994; Keller Peck et al., 2001; Nguyen, Parsadanian, Snider, & Lichtman, 1998; Zwick, Teng, Mu, Springer, & Davis, 2001). GDNF has also been shown to continually remodel the NMJ throughout adulthood (Zwick et al., 2001). Maintaining adequate levels of GDNF protein content in skeletal muscle, therefore, is important to preserve the neuromuscular system. Our results suggest that high intensity exercise provides trophic factor in fast twitch myofibers necessary to preserve fast motor units and thus slow the neurodegenerative changes associated with aging and disease.

Our laboratory and others provide substantial evidence that the regulation of GDNF expression is activity-dependent. The recruitment of the muscle seems to be an important factor when altering GDNF protein content within that given muscle. It is possible that the stress induced by muscle shortening or lengthening through contraction or stretch begins a cascade of events that upregulates GDNF expression downstream. This expression triggers a remodeling process that is crucial for the

survival and vitality of the motor neuron and innervating muscle fibers. The activity-dependent regulation, observed with GDNF expression, makes available trophic resources for those motor units that are being “used” and that are valuable in our day-to-day activities supporting the common phrase “use it or lose it”.

Summary

Exercise increases GDNF protein content and may act in coordination with the innervating motor neurons to nourish and strengthen the entire neuromuscular system during the aging and disease process. This will help to deter motor neuron degeneration and subsequent muscle wasting which is responsible for delaying the onset of frailty, dependence, morbidity and mortality observed in physically active individuals (Yarasheski et al., 1999).

Determining an exercise prescription that optimizes GDNF expression is an important direction for future research. Determining the optimal mode, intensity, duration, and frequency of exercise to optimize the health of the neuromuscular system can help deter some of the early onset motor unit wasting that is observed with hypokinetic and neurodegenerative diseases. Furthermore, in order to help “square off” the morbidity curve, early prescription for prevention of motor unit denervation is essential as it has been observed to begin as early as the third decade of life.

As Mark Twain pointed out, “life would be infinitely happier if we could only be born at the age of 80 and gradually approach 18”. This may not be necessary as

lifelong neurotrophic support through exercise can help achieve a fit and healthy independent lifestyle even into our ninth decade of life (Fiatarone et al., 1990).

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APPENDIX A

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC)

WESTERN MICHIGAN UNIVERSITY

Institutional Animal Care and Use Committee

Centennial 1903-2003 Celebration

Date: January 13, 2012

To: John Spitsbergen, Principal Investigator

From: Robert Eversole, Chair

Re: IACUC Protocol Number 12-01-03

Thank you for submitting your protocol with the required revisions. Your revised protocol entitled titled "Activity Dependent Regulation of Neurotrophic Factor Expression in Skeletal Muscle" has received approval from the Institutional Animal Care and Use Committee. The conditions and duration of this approval are specified in the Policies of Western Michigan University. You may now begin to implement the research as described in the application.

The Board wishes you success in the pursuit of your research goals.

Approval Termination: January 13, 2013

APPENDIX B

PUBLICATION #1-NEUROSCIENCE (CH. 2)

Neuroscience 257 (2014) 111–118

GLIAL CELL LINE-DERIVED NEUROTROPHIC FACTOR (GDNF) EXPRESSION AND NMJ PLASTICITY IN SKELETAL MUSCLE FOLLOWING ENDURANCE EXERCISE

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Abstract—Glial cell line-derived neurotrophic factor (GDNF) supports and maintains the neuromuscular system during development and through adulthood by promoting neuroplasticity. The aim of this study was to determine if different modes of exercise can promote changes in GDNF expression and neuromuscular junction (NMJ) morphology in slow- and fast-twitch muscles. Rats were randomly assigned to a run training (run group), swim training (swim group), or sedentary control group. GDNF protein content was determined by enzyme-linked immunosorbant assay. GDNF protein content increased significantly in soleus (SOL) following both training protocols ($P < 0.05$). Although not significant, an increase of 60% in the extensor digitorum longus (EDL) followed swim-training (NS; $P < 0.06$). NMJ morphology was analyzed by measuring α-bungarotoxin labeled post-synaptic end plates. GDNF content and total end plate area were positively correlated. End plate area decreased in EDL of the run group and increased in SOL of the swim group. The results indicate that GDNF expression and NMJ morphological changes are activity dependent and that different changes may be observed by varying the exercise intensity in slow- and fast-twitch fibers.

Key words: neuromuscular junction, fast-twitch muscle, slow-twitch muscle, exercise physiology, skeletal muscle, neuroplasticity.

INTRODUCTION

Maintaining a healthy neuromuscular system requires a continual supply of neurotrophic factors (NFs) to support developing and mature motor neurons. NFs support motor neurons throughout the lifespan by supporting maturation during embryonic life, maintenance through adulthood, and regeneration after injury (Oppenheim et al., 1995; Trupp et al., 1995; Yan et al., 1995; Keller-Peck et al., 1997; Keller Peck et al., 2001; Zwick et al., 2001; Wang et al., 2002). NFs have been shown to act alone and synergistically in their role to promote proper development and plasticity of adult and aged neurons. Of the many NFs identified to date, glial cell line-derived neurotrophic factor (GDNF) has been shown to be the most potent trophic factor for motor neuron survival *in vitro* (Lin et al., 1993; Henderson et al., 1994) and *in vivo* (Henderson et al., 1994). GDNF has been classified as belonging to the transforming growth factor superfamily and has been found to be a muscle-derived factor that regulates pre- and post-synaptic plasticity (Lin et al., 1993).

GDNF abides by the neurotrophic theory by being expressed in target skeletal muscle tissue, by being retrogradely transported to axonal cell bodies (Yan et al., 1995), and by providing survival support for motor neurons throughout their lifespan (Keller Peck et al., 2001). When compared to other NFs, GDNF has been shown to be up to 2500-fold more potent, being able to save nearly 100% of axotomized motor neurons and was the only factor to prevent axotomy-induced motor neuron atrophy (Henderson et al., 1994).

Although GDNF was first identified in midbrain dopaminergic neurons (Lin et al., 1993), it has since been found in numerous sites such as the skeletal muscle, Schwann cells, motor neuron axons and cell bodies (Henderson et al., 1994; Springer et al., 1994, 1995; Trupp et al., 1995; Nosrat et al., 1996). GDNF exerts its effects through the GDNF–GFR-α1–GPI complex, which interacts with Ret tyrosine kinase receptors that can be found localized presynaptically in the neuromuscular junction (Airaksinen and Saarma, 2002). The loss of Ret receptors decreases motor neuron survival embryonically, reduces adult motor neuron end plate numbers by half and results in a marked deficiency of maturing axon terminals within the adult muscle (Baudet et al., 2008). The overexpression of GDNF or treatment with GDNF has been shown to increase nerve terminal sprouting, slow the process of synapse elimination, and increase the number and size of end plates (Nguyen et al., 1998; Keller Peck et al., 2001; Zwick et al., 2001).

Neurotrophic factor secretion has been shown to increase following synaptic activity and may act to enhance transmission (Schinder and Poo, 2000). In the

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Abbreviations: ACh, acetylcholine; CSA, cross sectional area; EDL, extensor digitorum longus; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbant assay; GDNF, glial cell line-derived neurotrophic factor; NF, neurotrophic factor; NFs, neurotrophic factors; NMJ, neuromuscular junction; PBS, phosphate-buffered saline; SEM, standard error of the mean; SOL, soleus.

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CNS, NFs have been shown to exhibit enhanced or reduced expression and secretion following increased or decreased synaptic activity, respectively (Zafra et al., 1991; Lauterborn et al., 2000). The amount of NF secreted has also been shown to increase in proportion to the level of synaptic activity. The presence of secreted NFs, in turn, has been shown to induce a potentiation of transmitter release from presynaptic nerve terminals (Wang and Poo, 1997). Similar findings have been shown *in vitro* where NFs increase following chronic depolarization in culture (Lohof et al., 1993; Vianney and Spitsbergen, 2011) and *in vivo*, where NFs increase following exercise (Gómez Pinilla et al., 2002; Wehrwein et al., 2002; McCullough et al., 2011).

Exercise and GDNF independently have been shown to induce similar changes in the neuromuscular system, such as enhancing maintenance of synapses, inducing axonal sprouting (Andonian and Fahim, 1987; Keller Peck et al., 2001; Zwick et al., 2001), and increasing end plate complexity and size (Deschenes et al., 1993; Keller Peck et al., 2001). Exercise has been shown to induce synaptic activity and alter GDNF expression in the skeletal muscle and the spinal cord (Wehrwein et al., 2002; McCullough et al., 2011, 2013). Previous studies have shown that GDNF expression is regulated in an activity-dependent manner and its expression may depend on the recruitment of muscle fibers (Wehrwein et al., 2002; McCullough et al., 2011). For example, following low-intensity exercise, an increase of GDNF expression was observed in the SOL, a predominantly slow-twitch muscle, but decreased in the EDL, a predominantly fast-twitch muscle (McCullough et al., 2011). This may indicate that the low speed selected for the run training (10 m/min) was not of high enough intensity to recruit fast-twitch muscle fibers and consequently decreased GDNF protein content. Swim training has been shown to demand the recruitment of fast-twitch dorsiflexors more than run training, due to its higher cycling rate and to overcome the added resistance of the water medium during the recovery phase (Gruner and Altman, 1980; Roy et al., 1991). Swim training has shown similar recruitment patterns as run training in fast-twitch muscle fibers only when the treadmill reached higher speeds of ~67 m/min (Roy et al., 1991).

It is, therefore, our broad aim to examine GDNF expression and NMJ morphology in predominantly slow- and fast-twitch muscle fibers, following run and swim training. It is our hypothesis that the fast-twitch muscle fibers will be recruited and subsequently display an increase in GDNF protein content following swim training when compared to run training (10 m/min).

EXPERIMENTAL PROCEDURES

Subjects

Six-month-old Sprague Dawley rats (Charles River, Kalamazoo, MI, USA) were housed in rooms lighted from 7 AM–7 PM and given free access to standard rat chow and water *ad libitum*. Approval for this work was obtained from the Institutional Animal Care and Use Committee at the Western Michigan University. Rats were randomly assigned to a swim group ($n = 6$), run group ($n = 5$) or control group ($n = 6$). The control group was housed in individual standard living chambers and remained sedentary throughout the study.

Training protocols

The training protocol duration was based on previous studies that observed changes in GDNF expression following 2 weeks of exercise (McCullough et al., 2011, 2013).

Rats in the swim-train group (swim group) swam in a barrel (3/barrel) for 2 h/day for 2 weeks. Each swim consisted of five 24-min bouts separated by 10-min rest times. The water was filled to a depth of 100 cm and maintained at 35 °C, which was large enough for each rat to swim freely as previously published (Li et al., 2012; McCullough et al., 2013).

Rats in the run train group (run group) were placed in forced running wheels (Lafayette Instruments, Lafayette, IN, USA) for 2 h/day for 2 weeks as previously published (McCullough et al., 2013). The wheels were set at 10 m/min and each run consisted of five 24-min bouts separated by 10-min rest times to match the swim exercise.

Tissue preparation and storage

At the conclusion of the 2-week training period, rats from all groups were weighed and then euthanized via CO_2 asphyxiation and thoracotomy. Immediately following death, the soleus (SOL) and the extensor digitorum longus (EDL) muscles were removed. The muscles from the left side of the body were used for immunohistochemical analysis. Muscles were washed with phosphate-buffered saline (PBS: 0.225 M NaCl, 0.02 M NaH_2PO_4, and 0.08 M Na_2HPO_4), frozen at resting length in isopentane and stored at −80 °C. The muscles from the right side of the body were used to determine GDNF protein content using enzyme-linked immunosorbent assay (ELISA), as previously published (McCullough et al., 2011). Briefly, muscles were dipped in liquid nitrogen, smashed into fine powder, and homogenized in sample processing buffer solution (0.55 M NaCl, 0.02 M NaH_2PO_4, 0.08 M Na_2HPO_4, 2 mM EDTA, 0.1 mM benzethonium chloride, 2 mM benzamidine, 20 KIU/ml aprotinin, 0.5% BSA (bovine serum albumin), and 0.05% Tween-20). Homogenates were centrifuged at 4 °C for 30 min and the resultant supernatant was decanted and stored at −80 °C.

Immunohistochemistry

To analyze GDNF localization and NMJ structure, the middle sections of the SOL and EDL were removed from connective tissue, embedded in optimum cutting temperature compound (Sakura Finetek, Torrance, CA, USA) and cut on a Leica microtome-cryostat. Tissues were cut horizontally at 60 μm for end plate and GDNF visualization and cut transversely at 10 μm for fiber-type

analysis. All sections were thaw mounted on HistoBond Microscope Slides (VWR, 195 International, Bridgeport, NJ, USA). To help sections adhere, slides were vacuum sealed and left overnight at 4 °C. Slides were then fixed using 4% paraformaldehyde diluted in PBS at room temperature for 1 h. After fixing, the tissue was incubated in buffer containing 5% normal serum from the host species for the secondary antibody. Primary antibodies were incubated on sections overnight in a humidified chamber. To identify GDNF protein, polyclonal rabbit anti-GDNF antibody was used (Santa Cruz Biotechnologies, Santa Cruz, CA, USA; 1:200). End plates were identified using α-bungarotoxin (1:200) directly conjugated to AlexaFluor 488®. All fiber-type specific primary antibodies were purchased from Developmental Studies Hybridoma Bank, University of Iowa. The following day, slides were washed in PBS and secondary antibodies were applied at a concentration of 1:500. Secondary antibodies were conjugated to AlexaFluor 488®, AlexaFluor 568®, or AlexaFluor 647®. Slides were washed a final time followed by the application of 1:1 glycerol:PBS and a glass coverslip for microscopy.

Quantification: end plate size

A confocal microscope (Zeiss LSM 510) was used to visualize and collect end plate images. Once it was determined that the end plate was within the longitudinal border of the myofiber, a z-series of scans constructed a detailed picture using a C-Apochromat 63×/1.2 water correction objective (Zeiss, LLC). Fifty random endplates were captured from the EDL and SOL muscles for morphological analysis from at least three animals per group. Each end plate was measured for total area, stained area, total perimeter and stained perimeter using ImageJ software as previously described (Deschenes et al., 2006). In brief, a box was drawn around each end plate with lines touching each side of the stained area to determine total area and total perimeter. Total area included the stained regions and non-stained regions interspersed within those clusters residing inside the drawn box. Total Perimeter included the length of the box drawn around the end plate clusters that encompassed the stained and nonstained regions. Threshold was used to draw a line around only the stained area and stained perimeter of the individual end plate clusters.

Quantification: muscle fiber cross sectional area (CSA)

Frozen transverse cross-sections (10 μm) were stained against myosin heavy chain for the determination of muscle fiber cross-sectional area (CSA) in the SOL and EDL muscles. Widefield fluorescence microscopy was used to analyze random samples of muscle fibers from the EDL and SOL muscles as previously published (Legerlotz et al., 2008). Muscle fibers (125–150) were analyzed from three different animals per group to determine average myofiber CSA.

Statistics

All statistical analysis was performed using SPSS statistical software. Descriptive statistics were calculated to define means and standard errors for all variables. The results were reported as the mean ± standard error of the mean (SEM). A one-way analysis of variance (ANOVA) and Tukey's post hoc test were used to assess the statistical significance among the different groups. Linear regression analysis was performed on the individual samples to evaluate the association between variables. Statistical differences were considered significant at $P < 0.05$.

RESULTS

Training alters GDNF protein content

An ELISA was used in order to detect GDNF protein changes following exercise protocols in the SOL and EDL muscle fibers when compared to sedentary controls. An increase in GDNF protein content was observed ($P < 0.05$) following both run and swim training in the SOL muscles when compared to controls (Table 1). GDNF protein content in the EDL muscle was mostly affected following swim training. Although the increase in the swim group was not significant, it was increased by 60% ($P < 0.06$) when compared to the control group and was significantly different ($P < 0.05$) when compared to the run group.

Training induces changes in weight and CSA of muscle

The animal's body weight, relative muscle weight and muscle fiber CSA were measured in order to observe any changes following the 2 weeks of training when compared to sedentary controls.

The animals showed a decrease in their body weight following 2 weeks of run training ($P < 0.05$). Following swim training both the body weight and the relative muscle weight of the EDL muscle were increased when compared to the run and control groups ($P < 0.05$; Table 2).

The changes observed in the CSA of the muscle fibers were similar to GDNF trends in that both training protocols induced changes in the SOL muscle (Table 3). The CSA of the SOL muscle fibers decreased significantly following both training protocols ($P < 0.05$).

Neither training protocol significantly altered the CSA of the EDL muscle fibers when compared to controls

Table 1. GDNF protein content (pg/mg tissue weight; means ± S.E.M) in the SOL and EDL muscles following exercise. GDNF content increased in the SOL following run and swim training and increased in the EDL following swim training compared to the run training group

	Soleus	EDL
Control	2.5 ± 2.2	4.5 ± 2.4
Run	7.6 ± 6.4*	4.0 ± 1.4
Swim	8.6 ± 4.5*	7.2 ± 3.0#

Symbols denote statistical significance ($P \leq 0.05$) compared to the control (*) and the run group (#).

Table 2. Rat body weight (g) and tissue weight (mg; SOL & EDL) were measured in sedentary control animals as well as following 2 weeks of run and swim training

		Body Wt (g)	Tissue Wt (mg)	Relative muscle Wt (mg/g)
SOL	Control	459 ± 10	298 ± 19	0.65 ± 0.04
	Run	408 ± 11$^{*,\#}$	243 ± 31	0.65 ± 0.06
	Swim	453 ± 10	267 ± 23	0.59 ± 0.05
EDL	Control	459 ± 10	238 ± 18	0.52 ± 0.04
	Run	408 ± 11$^{*,\#}$	247 ± 21	0.62 ± 0.03
	Swim	453 ± 10	302 ± 20*	0.67 ± 0.04*

Symbols denote statistical significance ($P \leqslant 0.05$) compared to the control ($*$) and the swim group (#).

Table 3. Muscle fiber cross sectional area (CSA; means ± S.E.M) in the SOL and EDL muscles following exercise. CSA increased in the SOL following run and swim training and increased in the EDL of the swim group compared to the run group

	Soleus	EDL
Control	3831 ± 180	2574 ± 98
Run	2109 ± 65$^{*,\#}$	2352 ± 103
Swim	3243 ± 101*	2679 ± 104$^{\#}$

Symbols denote statistical significance ($P \leqslant 0.05$) compared to the control ($*$) and the swim group (#).

(Table 3). Although the CSA of the EDL muscle showed an increased trend, it was only significantly different from the run group ($P < 0.05$).

Training alters end plate morphology in SOL muscle fibers

The end plates of the SOL and EDL muscle fibers were measured to determine any changes in morphology following 2 weeks of training when compared to sedentary controls.

After 2 weeks of run training, no changes were observed for end plate area and size in the SOL muscle fibers. Run training did have an effect on measured perimeters as an increase was observed following the 2 weeks of training when compared to sedentary controls ($P < 0.05$; Fig. 1). End plate plasticity was observed following 2 weeks of swim training in the SOL muscle in all selected measurements ($P < 0.05$; Fig. 1). Increases in the end plate area and total perimeter were found following 2 weeks of swim training when compared to the run and control groups as well as an increase in the stained perimeter when compared to the control group.

Training alters end plate morphology in EDL muscle fibers

Two weeks of run training altered the end plate morphology of the EDL end plates by significantly reducing the stained area, total area, and total perimeter measurements when compared to sedentary controls ($P < 0.05$; Fig. 2). There were no differences observed in the end plates of the swim group when compared to controls following 2 weeks of swim training. Although end plate measurements were not significantly different from controls, this may indicate a resistance to atrophy that was not observed following run training.

GDNF protein content correlates with end plate total area

Correlation statistics were run to determine if a relationship exists between GDNF protein content and end plate measurements. GDNF protein content and end plate total area averages for all groups follow similar patterns of change (Fig. 3A). A relationship exists between GDNF protein content and end plate total area for all groups ($r = 0.611$, $P < 0.01$, $n = 30$; Fig. 3B). GDNF was observed in and around the end plates as well as separate from the end plates (Fig. 3C–E). This suggests that GDNF is expressed at the NMJ and in the muscle fibers as supported by the ELISA (Table 1). Increases in GDNF protein content and end plate area were observed in the SOL muscle following training (Fig. 3C–E).

DISCUSSION

GDNF expression & NMJ plasticity in soleus

One of the main findings of this study was the ability of the soleus muscle to increase GDNF protein content and exhibit significant plasticity at the NMJ following 2 weeks of run and swim training. Both training protocols were sufficient to induce an increase in GDNF protein content, further supporting an activity-dependent mechanism for neurotrophic support (McCullough et al., 2011). The observation that SOL muscle fiber CSA decreases with these exercise plans suggests that these muscles were recruited with these activities (Waerhaug et al., 1992; Deschenes et al., 1993, 2006). The training-induced reduction in fiber diameter is an indication of recruitment and is thought to be a positive adaptation to more readily exchange oxygen, carbon dioxide and waste products prolonging time to fatigue (Hoppeler et al., 2003).

The changes in the CSA of the SOL muscle fibers were most significant following the run training when compared to the swim and control groups possibly due to the differences in recruitment patterns of the two exercises selected. The two protocols place different demands on the recruitment patterns (Ariano et al., 1973) of the homogeneous slow-twitch SOL muscle (Armstrong and Phelps, 1984) and the fast-twitch EDL

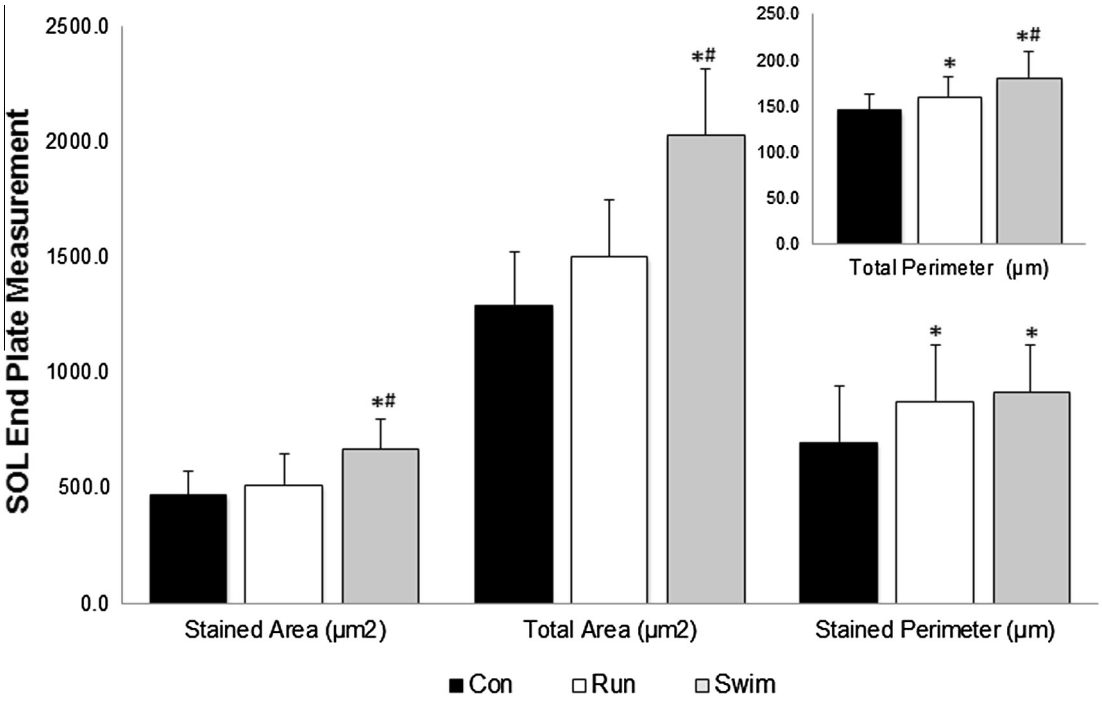

Fig. 1. Effects of exercise on post-synaptic endplate morphology in SOL muscle. Cross sections (60 μm) of SOL were stained with α-bungarotoxin for the visualization of motor endplates. Stained and total area (μm^2) increased significantly in the swim group (gray) when compared to the run group (white) and control group (black). Stained and total perimeter (μm) increased significantly in both exercise groups when compared to the control. Values are displayed as the mean ± SEM. Asterisk (∗) indicates a significant ($P \leqslant 0.05$) difference from the control group. Number symbol (#) indicates a significant ($P \leqslant 0.05$) difference from the run group.

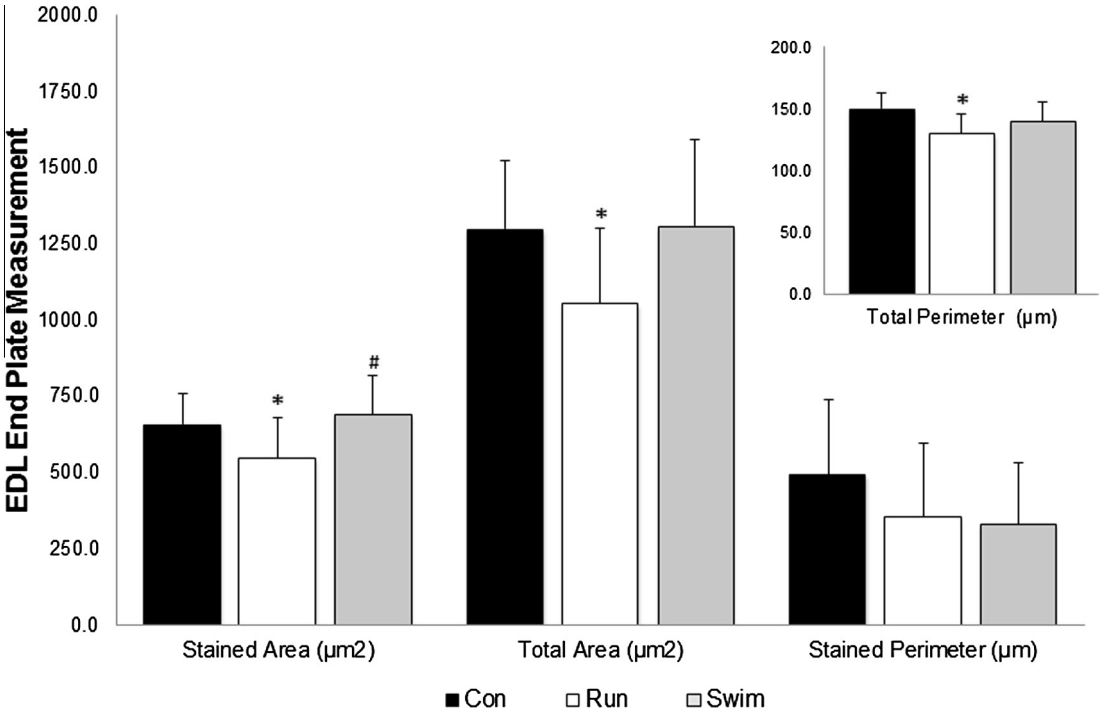

Fig. 2. Effects of exercise on post-synaptic endplate morphology in EDL muscle. Cross sections (60 μm) of EDL were stained with α-bungarotoxin for the visualization of motor endplates. Stained and total area (μm^2) and total perimeter (μm) increased significantly in the run group (white) when compared to the control group (black). Stained area (μm^2) for the swim group (gray) increased significantly from the run group. Values are displayed as the mean ± SEM. Asterisk (∗) indicates a significant ($P \leqslant 0.05$) difference from the control group. Number symbol (#) indicates a significant ($P \leqslant 0.05$) difference from the run group.

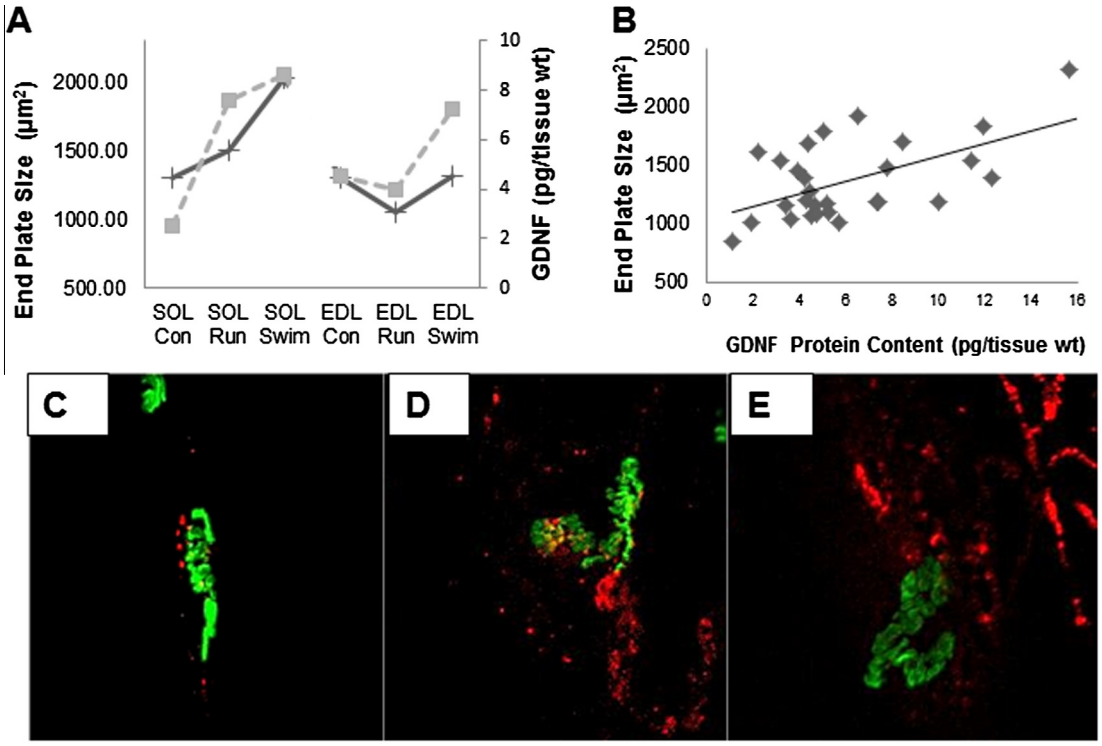

Fig. 3. Correlation between GDNF levels in skeletal muscle and total end plate area. End plates were visualized with α-bungarotoxin and measurements were taken using ImageJ software. Tissues were processed for GDNF protein content using ELISA and visualized by staining with anti-GDNF antibodies (blue). End plate total area (µm^2) was measured by drawing a box around the entire end plate, including the stained ACh receptor clusters and the area in between. (A) Average measurements showing the overall trend for all groups, comparing GDNF protein content (dashed line) and end plate total area (solid line). (B) Levels of GDNF (pg/mg tissue wt.) were positively correlated with end plate measurements for total area (r = .611, P < 0.01, n = 30). (C–E) Visualization of GDNF was labeled with rabbit anti-GDNF (blue) in and around SOL end plates labeled with α-bungarotoxin (green) in control (C), run (D), and swim groups (E).

muscle (Ariano et al., 1973). The low-intensity speeds selected for the run-training protocol have been shown to nearly maximize the recruitment of the slow-twitch SOL muscle fibers while higher cycling rates, such as swimming (more steps per unit time) may decrease that activation (Roy et al., 1991).

Both training protocols were able to alter the post-synaptic apparatus of the SOL muscle, consistent with previous findings supporting exercise as a means to alter end plate morphology in fully mature rats (Waerhaug et al., 1992; Deschenes et al., 1993; McCullough et al., 2011). GDNF protein content and end plate total area in the SOL were found to be positively correlated following run training consistent with previously findings (McCullough et al., 2011). The total end plate area may increase following presynaptic changes, in order to avoid fatigue (Hill et al., 1991). This activity-dependent adaptive mechanism may increase neurotransmitter stores (Stephens and Taylor, 1972) and quantal storage and release (Dorlöchter et al., 1991). This may subsequently demand the enlargement of its counterpart, the acetylcholine (ACh) receptor field. An increase in post-synaptic ACh receptor field has been previously shown following overexpression of GDNF (Keller Peck et al., 2001; Zwick et al., 2001; Wang et al., 2002).

GDNF expression & NMJ plasticity in EDL

The second substantial finding in this study was the ability of swim-training to increase GDNF protein content in fast-twitch muscle fibers by 60% compared to controls. Although this was not found to be statistically significant, it may be physiologically relevant and clinically important. These findings may provide encouragement for future studies to fine tune exercise prescription in order to recruit and support fast-twitch muscle fibers and their innervating neurons through expression of neurotrophic factors.

The EDL was presumably recruited as evidence of hypertrophy of the EDL muscle fibers based on the mean absolute and relative muscle weight gain following swim training. It has been previously shown the increased cycling rates in swimming escalate the demands for additional power via recruitment of larger fast-twitch fibers (Henneman and Mendell, 1981) and may be responsible for the trend toward an increase in GDNF content. Given the additional demand to meet the higher intensity swim-training and the resistance to dorsiflexion offered by the water medium, the fast-twitch EDL muscle has been shown to be more heavily recruited than the slow SOL muscle during swim-training. These results suggest that with further increases in intensity, such as resistance training, a

statistically significant increase in GDNF protein content in fast-twitch fibers may result.

This finding may be clinically significant given that sarcopenia, the age-associated decline in skeletal muscle mass and strength, is closely associated with increased frailty in the elderly, with decreased functional mobility and independence leading to a diminished quality of life, morbidity and mortality (Aniansson et al., 1984; Nevitt et al., 1989; Roubenoff, 2001). Sarcopenia appears to be fiber-type specific as large motor units that innervate fast-twitch skeletal muscle present as the most vulnerable to denervation, followed by intermediate and finally the most resilient small motor units (Frey et al., 2000; Doherty, 2003; Edström et al., 2007). The denervation of large motor units causes terminal sprouting of adjacent surviving motor units to muscle fibers left without innervation (Rich and Lichtman, 1989). This increases the size of the remaining motor units and may cause overburdening and exhaustion of that neuron, progressively leading to further denervation and eventual disability and mortality.

Denervation has been shown to occur before myofiber atrophy and is a necessary prerequisite for muscle mass loss and fiber-type conversion (Deschenes et al., 2010). Therefore, it has been suggested that by resisting the initial steps of denervation of the large motor units, the aging process of sarcopenia may be delayed in the neuromuscular system (Deschenes et al., 2010). In addition, significant changes have been observed in fast-twitch muscle fibers after disuse (Grimby et al., 1980) suggesting that exercise may play a key role in delaying the onset of aging in recruited fibers and in the neuromuscular system.

Both GDNF and exercise independently have been shown to protect large motor neurons from degeneration. GDNF-treated mice increased the large labeled motor neurons by 18-fold compared to β-Gal-treated mice and threefold more than untreated mice in a transgenic amyotrophic lateral sclerosis model (Mohajeri et al., 1999). Further studies are needed to determine if manipulation of exercise prescription can significantly increase GDNF protein content in fast-twitch muscle fibers, leading to protection of large motor neurons.

CONCLUSION

These findings support that GDNF is activity dependent and that it may be possible to increase GDNF's protein content in slow- and fast-twitch muscle fibers following different modes and intensities of exercise. It also supports the idea that GDNF plays a role in remodeling the NMJ in slow- and fast-twitch muscle fibers. This continues to be encouraging for the use of exercise as a mechanism to increase GDNF protein content in the skeletal muscle, leading to enhanced plasticity of the NMJ and enhanced neuromuscular health. With further research, guidelines can carefully inform exercise prescription that maximizes the neuromuscular benefits in both slow- and fast-twitch muscle fibers.

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APPENDIX C

PUBLICATION #2-PHYSIOLOGICAL REPORTS (CH. 3)

Physiological Reports

Open Access

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ORIGINAL RESEARCH

GDNF content and NMJ morphology are altered in recruited muscles following high-speed and resistance wheel training

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Keywords
Fast-twitch, GDNF, NMJ, skeletal muscle, voluntary exercise.

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Abstract

Glial cell line-derived neurotrophic factor (GDNF) may play a role in delaying the onset of aging and help compress morbidity by preventing motor unit degeneration. Exercise has been shown to alter GDNF expression differently in slow- and fast-twitch myofibers. The aim was to examine the effects of different intensities (10, 20, ~30, and ~40 m·min^{-1}) of wheel running on GDNF expression and neuromuscular junction (NMJ) plasticity in slow- and fast-twitch myofibers. Male Sprague-Dawley Rats (4 weeks old) were divided into two sedentary control groups (CON4 week, $n = 5$ and CON6 week, $n = 5$), two involuntary running groups, one at a low velocity; 10 m/min (INVOL-low, $n = 5$), and one at a higher velocity; 20 m/min (INVOL-high, $n = 5$), and two voluntary running groups with resistance (VOL-R, $n = 5$, 120 g), and without resistance (VOL-NR, $n = 5$, 4.5 g). GDNF protein content, determined by enzyme-linked immunosorbent assay (ELISA), increased significantly in the recruited muscles. Plantaris (PLA) GDNF protein content increased 174% ($P < 0.05$) and 161% ($P < 0.05$) and end plate-stained area increased 123% ($P < 0.05$) and 72% ($P < 0.05$) following VOL-R, and VOL-NR training, respectively, when compared to age-matched controls. A relationship exists between GDNF protein content and end plate area ($r = 0.880$, $P < 0.01$, $n = 15$). VOL-R training also resulted in more dispersed synapses in the PLA muscle when compared to age-matched controls ($P < 0.05$). Higher intensity exercise (>30 m/min) can increase GDNF protein content in fast-twitch myofibers as well as induce changes in the NMJ morphology. These findings help to inform exercise prescription to preserve the integrity of the neuromuscular system through aging and disease.

Introduction

The term sarcopenia was first coined in 1989 by Rosenberg to describe the age-related decline in skeletal muscle mass (Rosenberg 1997). It has since been shown that muscle mass alone may be a weak predictor of disability and mortality (Visser et al. 2005; Goodpaster et al. 2006; Newman et al. 2006). A relationship exists between the loss of muscle mass and loss of strength, but it is not linear due to a faster progression of the latter, leading to diminished muscle quality (muscle strength per unit of muscle mass) (Goodpaster et al. 2006; Delmonico et al. 2009). It has been suggested that muscle quality is a much better independent predictor of functional decline and mortality than loss of muscle mass alone and attention to this factor may be vital to delaying morbidity in the aging adult (Visser et al. 2005; Goodpaster et al. 2006; Newman et al. 2006).

One potential underlying cause of diminished muscle quality is the plasticity of the aging neuromuscular system, as seen with changes in motor units (MUs), peripheral nerves, and the neuromuscular junction (NMJ). As with loss of muscle mass and strength, a reduction of 1% of total MUs per year can be seen beginning as early as the third decade of life and this loss increases exponentially through the sixth decade (Tomlinson and Irving 1977). It has been predicted that between the age of 20–90, an 80% loss of MUs occurs with an accelerated rate of loss after the age of 60 (Wang et al. 1999). Many studies on human and animal aging have shown a preferential loss of the largest and fastest conducting MUs (Hashizume et al. 1988; Knox

et al. 1989; Ansved and Larsson 1990; Wang et al. 1999) causing atrophy of type II myofibers more so than type I (Fiatarone et al. 1990; Klitgaard et al. 1990; Larsson et al. 1991). While the mean cross-sectional area (CSA) of type I myofibers exceeds that of type II by 20% in the third and fourth decades of life (Brooke and Engle 1969), by the eighth decade of life, type II CSA is less than 50% of that of type I (Tomonaga 1977).

As a compensatory mechanism to maintain force production, collateral sprouting of nearby surviving motor axons reinnervate some, but not all type II myofibers (Pestronk et al. 1980). This causes the surviving MUs to increase in size leading to declines in force steadiness and fine motor control (Tracy et al. 2004), declines in motor conduction velocities in peripheral nerve (Wang et al. 1999), and a decline in MU firing rate at effort levels relevant to activities of daily living (Ling et al. 2009).

Physical activity, and specifically resistance training, has been found to be a reliable treatment to slow or reverse the declines observed in skeletal muscle through the aging process (Peterson et al. 2010). Older sedentary individuals have been found to be twice as likely to develop severe sarcopenia compared to age-matched active individuals (Janssen et al. 2002). Resistance training has been found to significantly increase muscle strength, mass, and functional mobility equally in both men and women (Leenders et al. 2013) and even into the ninth decade of life (Fiatarone et al. 1990). In addition, resistance training has the ability to upregulate protein synthesis as much as 50% in mixed myofibers in response to progressive overload (Yarasheski et al. 1999) and to induce type II myofiber hypertrophy by initiating satellite cell proliferation, differentiation, and fusion of new myonuclei into existing myofibers (Leenders et al. 2013). Furthermore, only resistance training versus a low intensity run- and swim-training had the ability to maintain the mean fiber area of large type II b fibers similar to young controls (Klitgaard et al. 1990).

One possible mechanism underlying the positive adaptations observed following resistance training in type II myofibers is the activity-induced protection of large MUs. Denervation of the motor neuron has been shown to precede, and to be a necessary prerequisite for, atrophy of the innervated myofiber (Deschenes et al. 2010). In addition, it has been postulated that the net loss of myofibers in sarcopenia may be due to an impaired capacity for axonal reinnervation of denervated myofibers (Aagaard et al. 2010). Therefore, if higher intensity exercise can recruit type II myofibers, perhaps the activity-induced protection of large MUs can prevent atrophy, and loss of type II myofibers, slowing or delaying the aging process.

A likely candidate to promote the survival of MUs following exercise is a neurotrophic factor named glial cell line-derived neurotrophic factor (GDNF). GDNF is produced in target tissues of neurons, including skeletal muscle (Suzuki et al. 1998), and acts in a retrograde fashion to exert nourishing effects (Yan et al. 1995). GDNF has been shown to be the most potent neurotrophic factor in promoting the survival of motor neurons and is the only candidate known to date to prevent motor neuron atrophy (Lin et al. 1993; Henderson et al. 1994). It has also been shown to regulate presynaptic and postsynaptic plasticity with a number of effects pertinent to sarcopenia, including causing hyperinnervation by inducing terminal sprouting, providing continuous synaptic remodeling, increasing end plate complexity and size, and protecting large MUs from degeneration (Andonian and Fahim 1987; Lin et al. 1993; Mohajeri et al. 1999; Keller Peck et al. 2001; Zwick et al. 2001).

GDNF expression has been shown to increase following 2 weeks of training in an activity-dependent manner, but only in presumably recruited muscles (Wehrwein et al. 2002; McCullough et al. 2011). Low intensity walk training was able to increase GDNF in slow-twitch myofibers, but decreased GDNF in fast-twitch myofibers (McCullough et al. 2011). In addition, GDNF has been reported to increase following higher intensity exercise in fast-twitch muscle fibers when comparing swim verse run training (Gyorkos 2014). The difference in GDNF protein content, however, might have been due to the difference between modes of exercise rather than to intensity changes. In order to clarify that the intensity is the factor regulating GDNF in fast-twitch skeletal muscle, different intensities of exercise with the same mode of training must be compared. Therefore, it is our hypothesis that a higher intensity exercise, such as voluntary wheel training, will recruit fast-twitch myofibers and subsequently alter GDNF protein content as well as induce plasticity of the NMJ.

Methods

Animals

All animal experiments were performed in accordance with the "Guide for the Care and Use of Laboratory Animals" (National Research Council) and protocols were approved by the Institutional Animal Care, and Use Committee at Western Michigan University. Four-week old male Sprague-Dawley rats ($n = 30$) were housed with 12:12-h light-dark. Rats were randomly divided into two sedentary control groups (CON4 week, $n = 5$ and CON6 week, $n = 5$), two involuntary running groups, one at a low velocity; 10 m/min (INVOL-low, $n = 5$) and one at a higher velocity; 20 m/min (INVOL- high, $n = 5$), and two voluntary running groups, one with

resistance (VOL-R, $n = 5$, 120 g), and one without resistance (VOL-NR, $n = 5$, 4.5 g) (Table 1).

Voluntary training protocol

All rats were housed in a clear polycarbonate living chamber (19″ × 10.5″ × 8″). The chambers in the VOL-R and VOL-NR groups were attached to an activity wheel system (Lafayette Instrument, Lafayette, IN) that was freely accessible at all times throughout the study. Voluntary exercise was chosen as the mode of training because rats are internally motivated to run at higher intensities spontaneously, and do not need noxious stimuli or external motivators such as food to induce running (Sherwin 1998; Legerlotz et al. 2008). It also allows the rats to run at night when they are naturally active.

The activity wheels were 35 inches in diameter and attached to a braking system and a counter with an optical sensor that sent distance and velocity information to be stored on a computer. Running distance was calculated by multiplying the number of rotations of the activity wheel by the circumference of the wheel (1.1 m). The wheel activity was continuously recorded for the duration of the study.

No resistance was applied to the VOL-NR, but a load of 4.5 g was necessary to overcome the inertia of the wheel. Resistance was applied to the VOL-R and calibrated daily to ensure 120 g of load was added to each wheel continuously for the length of the study. Calibration was accomplished by hanging 120 g of known weight on the wheel bar furthest from the axis and precisely tuning the braking system until the wheel could not be displaced.

Involuntary training protocol

The involuntary running groups consisted of a low-velocity running group (INVOL-low; 10 m·day^{-1}, $n = 5$) and a higher velocity running group (INVOL-high; 20 m·day^{-1}, $n = 5$) both being forced to exercise in motorized wheels. The INVOL-low group ran for five 24 min bouts separated by 5 min rest times as previously published (McCullough et al. 2011; Gyorkos 2014), while the INVOL-high group ran for five 15 min bouts separated by 5 min rest periods.

Table 1. Study design summary.

	Number	Speed (m·min^{-1})	Duration (km·day^{-1})	Length of time (week)
CON4 week	5	–	–	2
CON6 week	5	–	–	2
INVOL-low	5	10	1.2	2
INVOL-high	5	20	1.5	2
VOL-R	5	30	1.5	2
VOL-NR	5	40	2.2	2

Summary of study design for sedentary and exercised rats. The CON4 week and CON6 week groups were housed individually and remained sedentary for 2 weeks. The exercise groups were also housed individually, but were exercised daily for 2 weeks on a voluntary (VOL-) or involuntary (INVOL-) basis.

Tissue collection and processing

At the completion of the 2 weeks of training, the rats were weighed and euthanized by CO_2, asphyxiation, and thoracotomy. Immediately, the soleus (SOL), plantaris (PLA), and extensor digitorum longus (EDL) were excised from the hindlimbs and weighed. These muscles were chosen in this study to determine any potential differences between predominately slow- (SOL) and fast-twitch (EDL and PLA) myofiber phenotypes (Ariano et al. 1973; Armstrong and Phelps 1984) following exercise.

The muscles on the left side of the body were frozen at normal length in isopentane on dry ice and stored at −80°C, and used for immunohistochemistry. The muscles on the right side of the body were further processed and used for detection of GDNF protein content via enzyme-linked immunosorbent assay (ELISA), as previously published (McCullough et al. 2011). Each muscle was dipped in liquid nitrogen, smashed into fine powder, and homogenized with sample processing buffer (0.55 mol/L NaCl, 0.02 mol/L NaH_2PO_4, 0.08 mol/L Na_2HPO_4, 2 mmol/L ethylenediaminetetraacetic acid, 0.1 mmol/L benzethonium chloride, 2 mmol/L benzamidine, 20 KIU/mL aprotinin, 0.5% bovine serum albumin (BSA), and 0.05% Tween-20). Homogenates were centrifuged and supernatants were collected, and stored at −80°C.

Immunohistochemistry

The midbelly portions of the muscles were embedded in optimum cutting temperature compound (Sakura Finetek, Torrance, CA) and sectioned using a Leica microtome-cryostat. Sections were cut at 60 μm for visualization of GDNF localization and NMJ structure, and cut at 10 μm for fiber CSA quantification.

All sections were thaw mounted on HistoBond Microscope Slides (VWR; 195 International, Bridgeport, NJ), vacuum sealed, and stored overnight at 4°C.

Analysis of GDNF localization and NMJ structure

Rat muscle serial longitudinal sections (60 μm) were fixed in 4% paraformaldehyde at room temperature for 1 h, washed in phosphate buffered saline (PBS) (3 × 5 min), and incubated in buffer containing 10% donkey serum,

4% BSA, 0.5% Triton X-100 in PBS for 30 min in a humidified chamber at room temperature. Slides were then incubated with a primary polyclonal rabbit α-GDNF (Santa Cruz Biotechnologies; 1:200; Dallas, TX) overnight at 4°C. Slides were washed in PBS the next day and incubated for 2 h at room temperature in a secondary antibody cocktail including donkey α-rabbit conjugated to Alexa Fluor 647 (Molecular Probes, 1:1000; Eugene, OR) to visualize GDNF and α-bungarotoxin directly conjugated to AlexaFluor 488 (Molecular Probes, 1:1000) for end plate visualization. All antibodies were diluted in PBS containing 1% bovine serum albumin and 0.1% triton X-100. Slides were washed the next day in PBS and mounted in PBS: Glycerol (1:1) for microscopy. Images were viewed with a Zeiss Axiovert 100M confocal microscope (Zeiss LSM 510, Jena, Germany).

The sections stained with α-bungarotoxin were used for quantifying end plate measurements. Fifty random end plates were captured for each muscle (SOL, PLA, and EDL) in the study using the confocal microscope with a C-Apochromat 63×/1.2 water correction objective. Each end plate was visually analyzed to make certain that it was within the longitudinal border of the myofiber before being scanned and stored. Each captured end plate was analyzed using Image J software as previously described (Deschenes et al. 2006). Briefly, a box was drawn around each end plate with the lines of the box touching each side of the stained area. The area within the box represented the total area and total perimeter measurements of the end plate. This area included the stained as well as the nonstained area interspersed between the end plate clusters. The image was then thresholded to identify the α-bungarotoxin staining and represented the stained area and stained perimeter. Dispersion was measured by dividing the stained area by the total area of the end plate; therefore, a lower percentage would indicate a more dispersed synapse.

Determination: myofiber CSA

The myofiber CSA of the muscle fibers was determined as previously published (Legerlotz et al. 2008). Using wide-field fluorescence microscopy, 125–150 random muscle fibers were captured for each muscle (SOL, PLA, and EDL) in this study. Each muscle fiber was encircled and analyzed using Image J software (http://rsbweb.nih.gov/ij/).

Statistics

All statistical analyses were performed using SPSS statistical software (http://www-01.ibm.com/software/analytics/spss/). Descriptive statistics were calculated to define means and standard errors for all variables. The results were reported as the mean ± standard error of the mean (SEM). Data were analyzed using a one-way analysis of variance (ANOVA) and Tukey's post hoc comparison to test for statistical significant differences between groups. Linear regression analysis was performed on the individual samples to evaluate the association between variables. Differences were considered statistically significant at $P < 0.05$.

Results

Training

On a daily average, the VOL-NR group ran ~33% faster (~40 m·min^{-1} vs. ~30 m·min^{-1}; $P < 0.05$) and ~46% longer (~2.2 km·day^{-1} vs. ~1.5 km·day^{-1}; $P < 0.05$) than the VOL-R group. The amount of work averaged over the 2 weeks of training was similar between groups when factoring in the 120 g of resistance added to the VOL-R group using a previously published equation: W = force (N) × distance (msec)/body wt (kg) (Ishihara et al. 1998). The involuntary running protocols matched closely in running distance for INVOL-low (10 m·min^{-1}) and INVOL-high groups (20 m·min$^{-1)}$ as they ran for 1.2 km·day^{-1} and 1.5 km·day^{-1}, respectively.

Animal body weight and muscle weights

Body weight decreased ($P < 0.05$) following all of the exercise training protocols with the exception of VOL-R training (Table 2). Although, the absolute tissue weight was significantly altered in all three muscle groups (SOL, PLA, EDL) depending on the exercise, only the relative weight of the PLA muscle was increased ($P < 0.05$) following voluntary exercise (VOL-R and VOL-NR). The relative weight of the SOL and EDL muscles remained unaltered following exercise protocols when compared to sedentary controls.

Effects of exercise on PLA muscle

GDNF protein content in PLA

CSA was measured as an indicator of muscle recruitment where slow- and fast-twitch myofibers have been shown to respond differently by decreasing and increasing CSA, respectively (Waerhaug et al. 1992; Deschenes et al. 1993, 2010). The CSA of the PLA myofibers increased significantly following both VOL-R and VOL-NR exercise when compared to age-matched sedentary controls (Fig. 1; bar graph). In those recruited muscles, GDNF protein content increased 174% ($P < 0.05$) following VOL-R exercise and 161% ($P < 0.05$) following VOL-NR exercise when

Table 2. Animal body and muscle weights (Values are means ± SEM).

	Body wt (g)	Tissue wt (mg)	Relative muscle wt (mg/g)
SOL			
Control	264 ± 12	149 ± 19	0.56 ± 0.04
INVOL-low	217 ± 14*	113 ± 32*	0.52 ± 0.05
INVOL-high	205 ± 15*	95 ± 28*	0.46 ± 0.07
VOL-R	272 ± 14	133 ± 23	0.49 ± 0.05
VOL-NR	230 ± 15*	128 ± 20*	0.55 ± 0.06
PLA			
Control	264 ± 12	234 ± 21	0.88 ± 0.03
INVOL-low	217 ± 14*	258 ± 19	1.10 ± 0.04
INVOL-high	205 ± 15*	240 ± 28	1.17 ± 0.06
VOL-R	272 ± 14	351 ± 18*	1.29 ± 0.03*
VOL-NR	230 ± 15*	293 ± 26*	1.27 ± 0.06*
EDL			
Control	264 ± 12	142 ± 18	0.54 ± 0.06
INVOL-low	217 ± 14*	119 ± 16*	0.57 ± 0.07
INVOL-high	205 ± 15*	125 ± 18*	0.57 ± 0.05
VOL-R	272 ± 14	158 ± 19	0.58 ± 0.04
VOL-NR	230 ± 15*	131 ± 26	0.57 ± 0.06

Rat body weight (g), absolute (mg), and relative tissue weights (mg/g) were measured in sedentary control animals and following training protocols. The relative muscle weight of the PLA increased following voluntary exercise (VOL-R and VOL-NR). Symbols denote statistical significance ($P \leq 0.05$) compared to control (*).

compared to age-matched sedentary controls (Fig. 1; line graph). There were no significant differences in PLA GDNF protein content or CSA between VOL-R and VOL-NR training groups, or following involuntary exercise compared to age-matched sedentary controls.

End plate measurements in PLA

Immunohistochemistry revealed that all of the measurements quantified for end plates in the PLA myofibers were altered following voluntary exercise. Total area, stained area, total perimeter, and stained perimeter were all increased in the VOL-R and VOL-NR groups compared to age-matched controls ($P < 0.05$; Fig. 2). The end plate stained area increased 123% ($P < 0.05$) and 72% ($P < 0.05$) following VOL-R and VOL-NR training, respectively, when compared to age-matched controls. Voluntary training groups showed increased end plate measurements compared to age-matched controls. The end plates increased even further following VOL-R training when compared to VOL-NR training. A positive relationship exists between PLA GDNF protein content and end plate area ($r = 0.880$, $P < 0.01$, $n = 15$) (Figs. 4, 5). VOL-R training also resulted in more dispersed synapses when compared to VOL-NR training and age-matched control groups ($P < 0.05$; Fig. 2 inset). There were no significant differences observed in end plate measurements following involuntary exercise compared to age-matched sedentary controls.

Effects of exercise on SOL muscle

GDNF protein content in SOL

The CSA of the SOL myofibers decreased following INVOL-low and VOL-NR training when compared to age-matched sedentary controls (Fig. 1; bar graph). SOL GDNF protein content increased 145% ($P < 0.05$) and 272% ($P < 0.05$) following INVOL-low and VOL-NR training, respectively, when compared to age-matched controls (Fig. 1; line graph). There were no significant differences observed in measurements of fiber CSA and GDNF following INVOL-high and VOL-R training compared to age-matched sedentary controls.

End plate measurements in SOL

The total and stained area and total perimeter of the SOL end plates increased following INVOL-low and VOL-NR training (Fig. 3). Stained area of SOL end plates increased 89% ($P < 0.05$) and 100% ($P < 0.05$) following INVOL-low and VOL-NR training, respectively, when compared to age-matched sedentary controls. There were no significant differences in dispersion of the SOL end plates. A relationship exists between GDNF protein content and end plate area ($r = 0.880$, $P < 0.01$, $n = 15$) (Figs. 4, 5). There were no significant differences observed in end plate measurements following INVOL-high and VOL-R training compared to age-matched sedentary controls.

Effects of exercise on EDL muscle

CSA and GDNF protein content in EDL

The CSA of the EDL myofibers decreased following VOL-NR training compared to age-matched sedentary controls (Fig. 1; bar graph). There were no other significant differences observed in measurements of fiber CSA and GDNF (Fig. 1; line graph) compared to age-matched sedentary controls.

End plate measurements in EDL

There were no significant differences observed among end plate measurements when compared to age-matched sedentary controls. There were no differences in the dispersion of the EDL end plates when compared to controls. A relationship exists between GDNF protein content and end plate area ($r = 0.880$, $P < 0.01$, $n = 15$) (Figs. 4, 5).

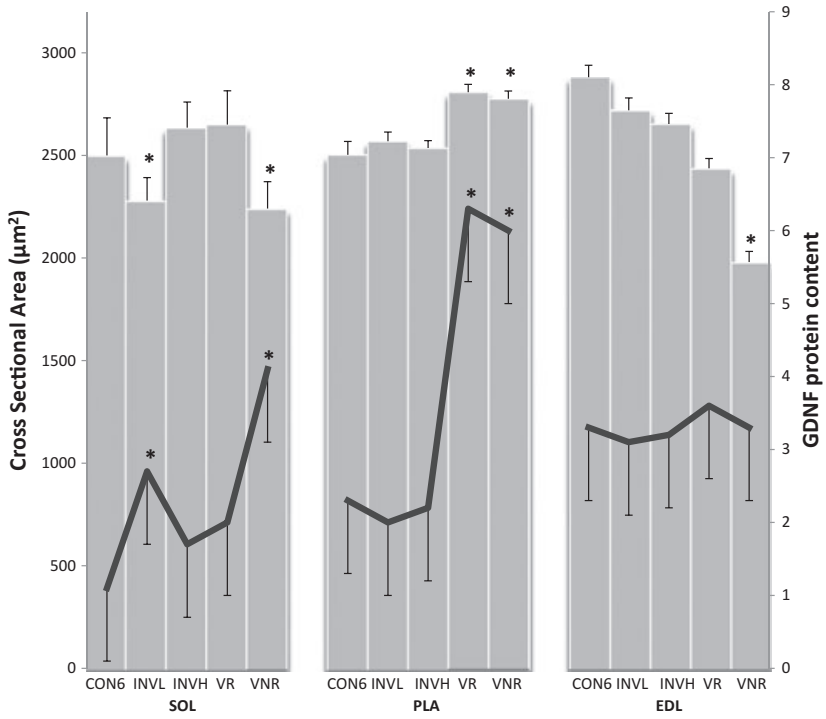

Figure 1. GDNF protein content increases in recruited muscles. Quantification of GDNF was accomplished via ELISA. Cross-sectional area (CSA; bar graph) was measured in 125–150 random SOL, EDL, and PLA fibers that were captured by widefield microscopy and analyzed using ImageJ software. GDNF protein content (pg/mg per tissue weight; line graph), determined by ELISA, was significantly increased in all recruited fibers of the SOL and PLA muscles, evidence by CSA decreasing and increasing, respectively. Increases in GDNF protein content were observed in SOL muscle following INVOL-low and VOL-NR training protocols. Increases in GDNF were observed in PLA muscle following both voluntary training. The EDL did not appear to be recruited following any training and no changes in GDNF were observed. Values are displayed as the mean ± SEM. Asterisk (*) indicates a significant ($P \leq 0.05$) difference from the age-matched control group. Key: CON6 (CON6 week), INVL (INVOL-low), INVH (INVOL-high), VR (VOL-R), and VNR (VOL-NR).

Discussion

This is the first study to our knowledge that has shown an increase in GDNF protein content in fast-twitch myofibers. GDNF levels in fast-twitch myofibers have been found to significantly decrease following short-term exercise at low velocities (10 m·day^{-1}) (McCullough et al. 2011). We were able to show that by increasing both the velocity (30–40 m·day^{-1}) and the resistance (120 g) of the training, the fast-twitch myofibers of the PLA muscle were recruited, and subsequently increased GDNF content.

Rats will spontaneously and naturally run at speeds close to their maximal aerobic capacity in short bouts mimicking sprint training (Shepherd and Gollnick 1976; Rodnick et al. 1989). It has been shown that the high-intensity nature of voluntary exercise can induce increases in enzymatic activity in fast-twitch myofibers and that these changes are unlikely caused by hormonal changes, but may be a result of different patterns of muscle recruitment following higher intensity training (Bagby et al. 1986; Rodnick et al. 1989). Speeds as low as 25–40 m/min have been found to significantly deplete glycogen, increase lactate accumulation and to increase cytochrome *c* concentration in fast-twitch myofibers (Armstrong et al. 1974; Baldwin et al. 1977; Dudley et al. 1982) indicating recruitment of those myofibers. In this study, rats were able to reach speeds between ~30 and 40 m/min on average through voluntary exercise, which reaches the intensity threshold for recruitment (Armstrong et al. 1974; Baldwin et al. 1977; Dudley et al. 1982).

Addition of a load of 120 g of resistance within the first 2 weeks of this study was enough to induce hypertrophy in the PLA muscle as previously reported (Ishihara et al. 1998; Legerlotz et al. 2008). An increase in the CSA of the PLA muscle has been shown to follow an increase in load (Ishihara et al. 1998; Legerlotz et al. 2008) or an increase in duration (Kariya et al. 2004) of the training period. The voluntary exercise reached an intensity that was high enough to recruit the fast-twitch myofibers in both training groups and may have contributed to an increase in GDNF protein content in the PLA muscle. This is clinically relevant as this indicates that higher

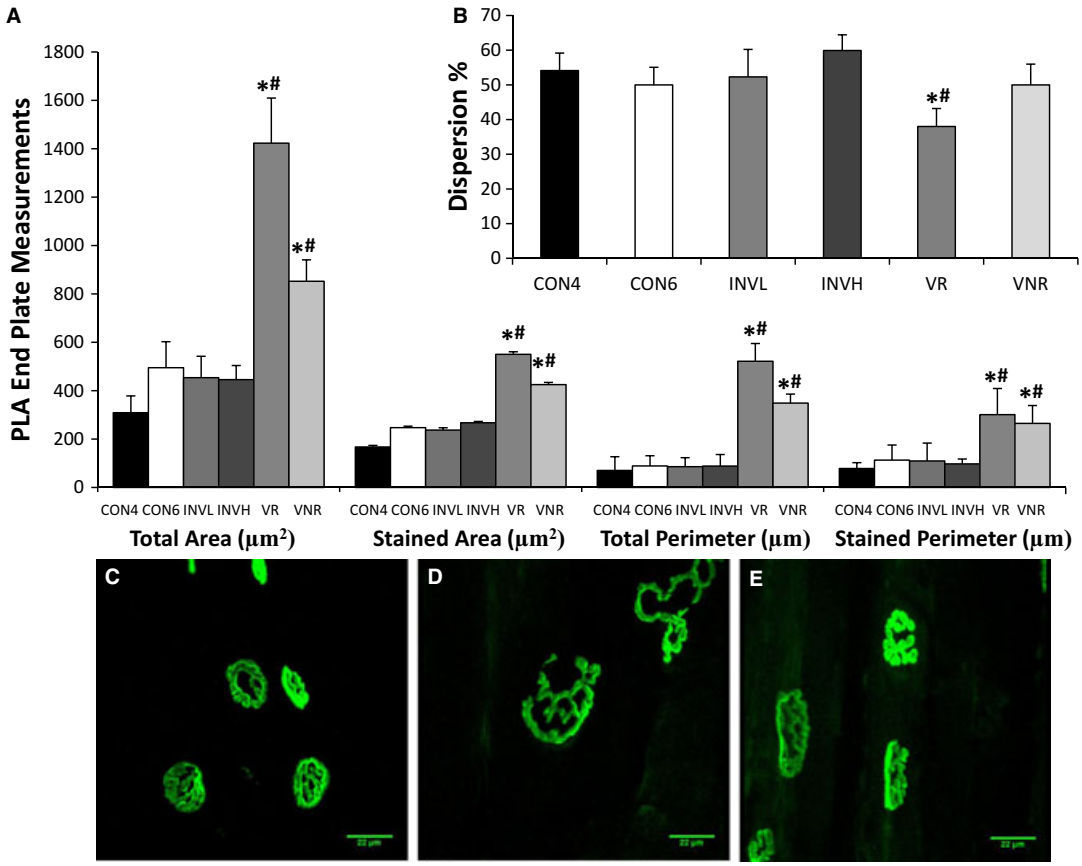

Figure 2. Effects of exercise on postsynaptic end plate morphology in PLA muscle. Cross sections (60 μm) of SOL muscle fibers were stained with α-bungarotoxin for quantification and visualization. Total and stained area (μm^2), and total and stained perimeter (μm) were altered following VOL-R and VOL-NR training when compared to age-matched sedentary controls (A). In addition, VOL-R training resulted in more dispersed synapses compared to the 6 week control group (B). End plates are shown for the 6 week-CON (C), VOL-R (D), and VOL-NR (E) groups. Note the dispersion observed following VOL-R training (D). Values are displayed as the mean $\pm$ SEM. Asterisk (*) indicates a significant ($P \leq 0.05$) difference from the age-matched sedentary control group. Number symbol (#) indicates a significant ($P \leq 0.05$) difference from the 4 week-control group. Key: CON4 (CON4 week), CON6 (CON6 week), INVL (INVOL-low), INVH (INVOL-high), VR (VOL-R), and VNR (VOL-NR).

intensity training may be able to support the innervating neurons of those vulnerable fast-twitch myofibers through increased expression of neurotrophic factors.

We were also able to show that the training altered the end plate morphology of the recruited muscles, consistent with previous findings that exercise can induce changes to the postsynaptic apparatus in rats (Waerhaug et al. 1992; Deschenes et al. 1993; McCullough et al. 2011). Deschenes et al. (1993) observed increases in the dispersion of end plates of fast-twitch myofibers following higher intensity training. This study showed similar results as the PLA end plates were more dispersed following VOL-R training, but not following VOL-NR training. It has been postulated that this pattern of dispersion following higher intensity exercise may be the result of longer and more complex arborization of the presynaptic terminal, compared to the shorter primary branches observed following lower intensity exercise (Deschenes et al. 1993). These results suggest that the intensity of exercise alters the NMJ of slow- and fast-twitch myofibers in different ways.

GDNF, in the absence of exercise, has also been found to cause continuous synaptic remodeling of the NMJ by inducing hyperinnervation, increasing end plate size, and complexity, and maintaining the postsynaptic apparatus (Keller Peck et al. 2001; Zwick et al. 2001; Wang et al. 2002). The results of this study are consistent with those findings as GDNF protein content was positively correlated with end plate size. It has been postulated that presynaptic changes, such as terminal sprouting, increased neurotransmission stores, and quantal storage and release may warrant changes in the postsynaptic apparatus in order to enhance communication and efficiency at the NMJ (Stephens and Taylor 1972; Dorlöchter et al. 1991; Hill et al. 1991; Keller Peck et al. 2001; Zwick et al. 2001).

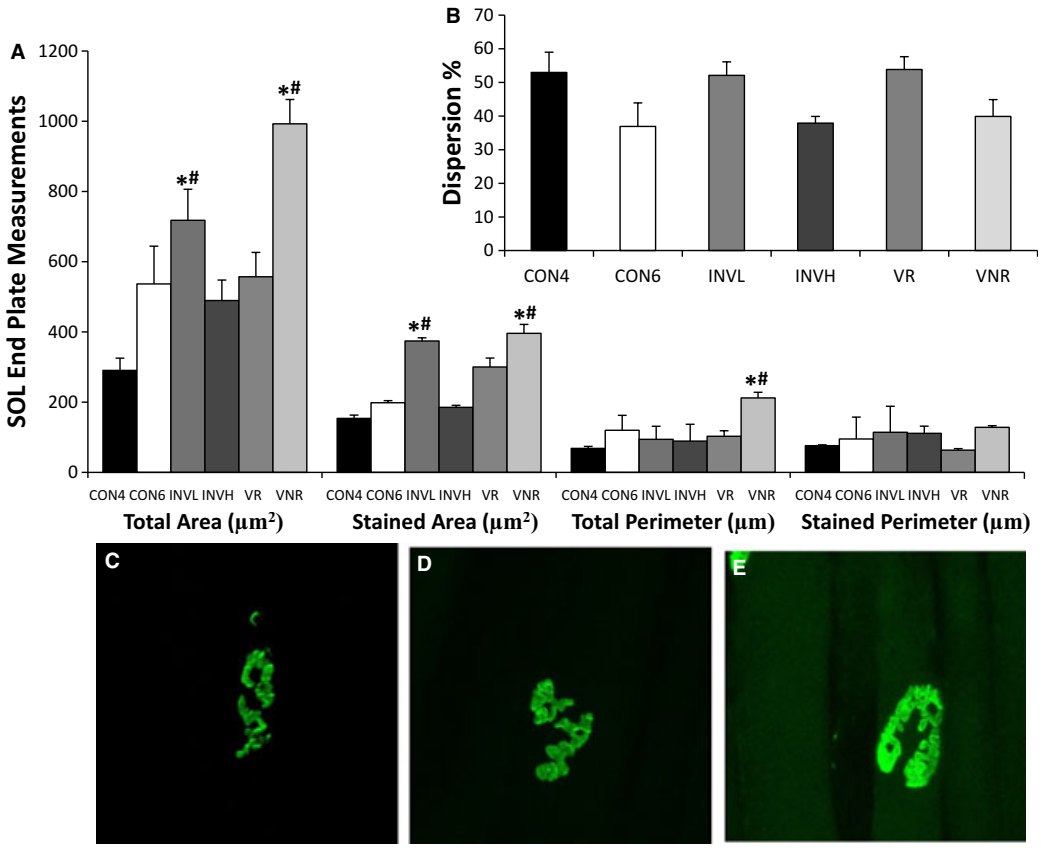

Figure 3. Effects of exercise on postsynaptic end plate morphology in SOL muscle. Cross sections (60 μm) of SOL muscle fibers were stained with α-bungarotoxin for quantification and visualization. Total and stained area (μm^2) were altered following INVOL-low and VOL-NR training when compared to age-matched sedentary controls (A). In addition, total perimeter was increased following VOL-NR training. No significant differences in dispersion were observed (B). End plates are shown for the 6 week CON (C), INVOL-low (D), and VOL-NR (E) groups. Values are displayed as the mean $\pm$ SEM. Asterisk (*) indicates a significant ($P \leq 0.05$) difference from the age-matched sedentary control group. Number symbol (#) indicates a significant ($P \leq 0.05$) difference from the 4 week control group. Key: CON4 (CON4 week), CON6 (CON6 week), INVL (INVOL-low), INVH (INVOL-high), VR (VOL-R), and VNR (VOL-NR).

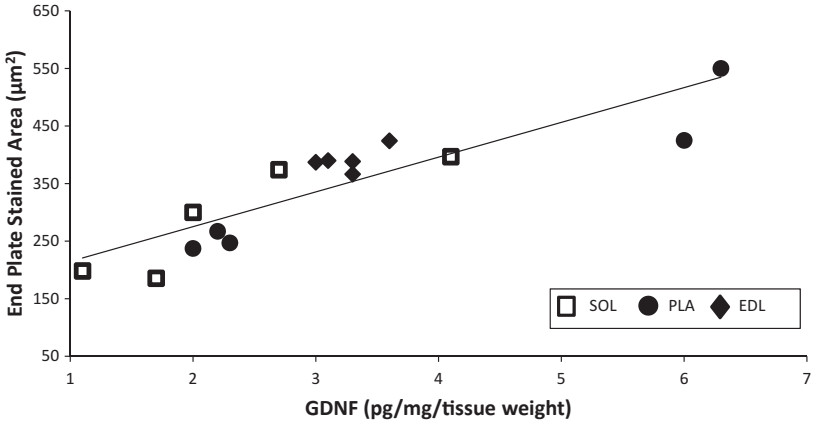

Figure 4. Correlation between GDNF levels in skeletal muscle and end plate-stained area. A relationship exists between GDNF protein content (pg/mg/tissue weight) and end plate-stained area (μm^2) ($r = 0.880$, $P < 0.01$). Each point represents one of the three muscles (SOL, PLA, EDL) in one of five groups (CON6 week, INVOL-low, INVOL-high, VOL-R, and VOL-NR), giving 15 data points ($n = 15$).

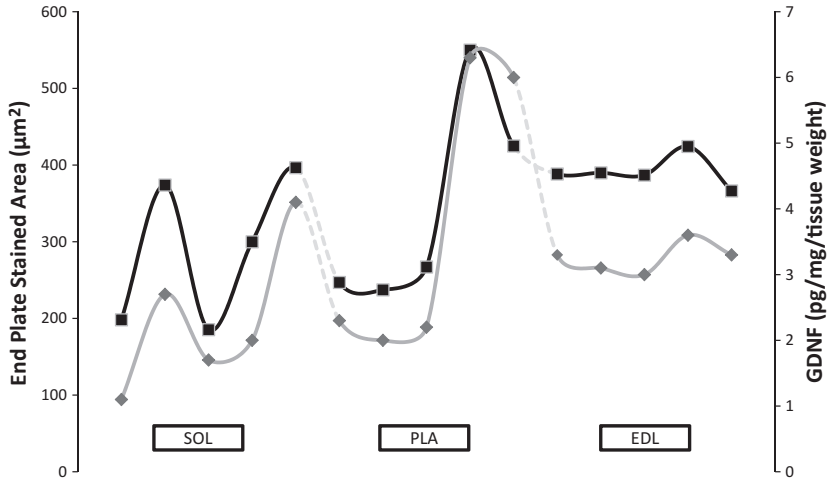

Figure 5. Relationship between GDNF protein content and end plate-stained area. This graph displays the close relationship between GDNF and end plate area in the SOL, PLA, and EDL muscles.

It is noteworthy that the muscles that were not actively recruited; PLA during involuntary training, SOL during INVOL-high, and VOL-R training, and EDL following all training, with the exception of VOL-NR, displayed no changes in NMJ morphology, and GDNF levels remained similar to those in sedentary controls. These findings provide further support that the regulation of GDNF is activity-dependent, as its expression relies on the recruitment of the myofibers during physical activity (Wehrwein et al. 2002; McCullough et al. 2011).

Physical activity levels and intensity decline with aging, with an associated decline in skeletal muscle mass and strength (Rosenberg 1997; Janssen et al. 2002). Those losses in mass and strength result in significant public health problems with associated increases in falls, fractures, and frailty, declines in functional mobility, and independence, leading to a diminished quality of life, morbidity, and mortality (Aniansson et al. 1984; Nevitt et al. 1989; Roubenoff 2001; Janssen et al. 2002). It has been postulated that there is no other decline in aging that is as dramatic or as significant as that of lean body mass (Rosenberg 1997). The decline of 3–8% of muscle mass per decade begins as early as 30 years of age with ~45% of the U.S. population sarcopenic and 20% functionally disabled as they approach 65 years of age (Manton and Gu 2001). The population over 60 years old is predicted to triple in the next 50 years and with it comes diminished quality of life and disability, leading to economic costs for government reimbursed health care, unless underlying mechanisms are identified and preventative measures are taken (Janssen et al. 2002).

Being physically active, on the other hand, has been shown to compress morbidity and have a "squaring off" effect on the disability and mortality curves so that people can live longer productive lives, and die after a limited period of disability (Fries 2002). In this context, exercise has been compared to a nonpharmacological fountain of youth (Joyner and Barnes 2013). In addition, physical fitness has been shown to be the number one independent risk factor for both all-cause and cardiovascular morbidity (Kodama et al. 2009).

GDNF is a muscle-derived, activity-dependent factor that may play a critical role in the positive outcomes of exercise. GDNF may act to promote the survival of MUs and, therefore, play a pertinent role in delaying the onset of aging of the neuromuscular system. The effects of exercise as a regulatory factor of GDNF expression deserves continued research in order to inform exercise prescription to induce changes in those myofibers that are most susceptible to degeneration, preserving nerve and muscle function, and protecting the neuromuscular system.

Conflict of Interest

None declared.

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Graphical Abstract

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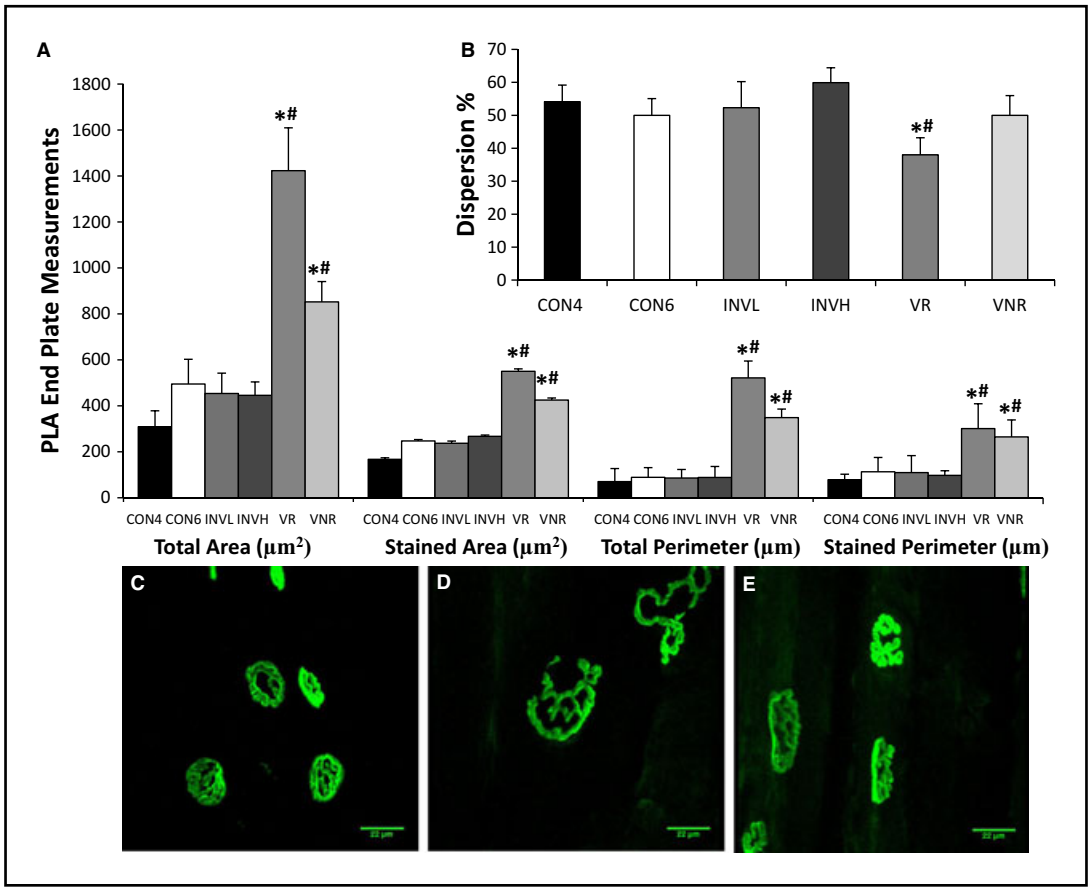

Higher intensity exercise (>30 m/min) can increase glial cell line-derived neurotrophic factor (GDNF) protein content in fast-twitch myofibers as well as induce changes in the neuromuscular junction (NMJ) morphology. These findings help to inform exercise prescription to preserve the integrity of the neuromuscular system through aging and disease.

APPENDIX D

PUBLICATION #3-NEUROSCIENCE

Neuroscience 240 (2013) 258–268

SHORT-TERM EXERCISE INCREASES GDNF PROTEIN LEVELS IN THE SPINAL CORD OF YOUNG AND OLD RATS

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Abstract—Neurotrophic factors may play a role in exercise-induced neuroprotective effects, however it is not known if exercise mediates changes in glial cell line-derived neurotrophic factor (GDNF) protein levels in the spinal cord. The aim of the current study was to determine if 2 weeks of exercise alters GDNF protein content in the lumbar spinal cord of young and old rats. GDNF protein was quantified via an enzyme-linked immunosorbent assay and Western blot. Immunohistochemical analysis localized GDNF in choline acetyltransferase (ChAT)-positive motor neurons and cell body areas were measured. Involuntary running in the young animals appeared to elicit the greatest increase in GDNF protein content (sixfold increase), followed by swimming (threefold increase) and voluntary running (twofold increase); however there was no significant difference between the modalities of exercise. Low-intensity running of the old animals significantly increased GDNF protein content in the spinal cord. Both young and old exercised animals showed a doubling in ChAT-positive motor neuron cell body areas. These results suggest that GDNF protein content in the spinal cord is modulated by exercise.

Key words: spinal cord, exercise, aging, neural plasticity, GDNF, motor neuron.

INTRODUCTION

A significant loss of skeletal muscle mass and strength are commonly observed in aging individuals (Kallman et al., 1990; Frontera et al., 2000) and contribute to an increased incidence of falls and disability (Fries et al., 1994; Toulotte et al., 2003). Changes with age are observed both in skeletal muscle and motor neurons innervating skeletal muscles. Alterations in motor neurons with increased age include loss of somatic motor neurons (Jacob, 1998) and loss of inputs to motor nerve cell bodies (Kullberg et al., 1998). In the aging rat there is a decrease in muscle innervation, loss of myelinated nerve fibers and changes in expression of neuropeptides and growth factors, similar to what is observed following axon lesion (Johnson et al., 1999). One possible contributing factor for the loss of motor neurons with age could be diminished neurotrophic factor signaling (Bergman et al., 1999).

Glial cell line-derived neurotrophic factor (GDNF) was first discovered in glial cells (Lin et al., 1993), and its expression has been found in a variety of tissues both in the central and peripheral nervous systems (Henderson et al., 1994; Suter-Crazzolara and Unsicker, 1994; Springer et al., 1995; Suzuki et al., 1998). To date, GDNF is the most potent survival factor identified for motor neurons (Henderson et al., 1994), where heterozygous GDNF knockout mice lack 22% of their lumbar motor neurons (Moore et al., 1996), and GDNF receptor alpha-1 (GFRα-1) knockout mice lack 24% of their lumbar motor neurons (Cacalano et al., 1998). One possible source of GDNF for somatic motor neurons is skeletal muscle, where GDNF is transported in a retrograde fashion (Yan et al., 1995; Trupp et al., 1997; Wang et al., 2002).

Increased expression of GDNF in developing skeletal muscle leads to increased axonal branching and increased motor unit size (Nguyen et al., 1998; Zwick et al., 2001), while treatment with exogenous GDNF causes continuous synaptic remodeling at the neuromuscular junction (Keller-Peck et al., 2001) and prevents motor neuron degeneration following axotomy (Oppenheim et al., 1995). GDNF increases choline acetyltransferase (ChAT) activity of embryonic motor neurons (Zurn et al., 1994), rescues somatic motor neurons from naturally occurring cell death (Oppenheim et al., 2000) and from axotomy-induced cell death (Oppenheim et al., 1995), and protects motor neurons from chronic degeneration (Corse et al., 1999). Neurotrophic factors, such as brain-derived neurotrophic factor (BDNF), insulin-like growth factor 1, and vascular endothelial growth factor (Trejo et al., 2001; Fabel et al., 2003; Wu et al., 2008) have been suggested to play a role in exercise-mediated neuroprotective effects, however it is not known if GDNF plays a similar role. While independent studies have found similar beneficial effects following exercise to those observed with exogenous treatment with GDNF, no one has been able to link the two together. One of the goals of our studies is to determine if the beneficial effects of exercise for

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Abbreviations: BDNF, brain-derived neurotrophic factor; ChAT, choline acetyltransferase; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; GDNF, glial cell line-derived neurotrophic factor; NGF, nerve growth factor; PVDF, polyvinylidene difluoride; SEM, standard error of the mean.

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the motor nervous system may, in part, be driven by changes in GDNF levels. Here, we report that short-term exercise increases GDNF protein content in the lumbar spinal cord of young (6-month-old) and old (24-month-old) rats, at the same time we observed morphological changes of motor neuron cell bodies.

EXPERIMENTAL PROCEDURES

Subjects

All experiments were performed in accordance with the ''Guide for the Care and Use of Laboratory Animals'' (National Research Council) and protocols were approved by the Institutional Animal Care and Use Committee at Western Michigan University. Male Sprague–Dawley rats (Charles River, Kalamazoo, MI, USA) were given access to food and water *ad libitum* and were maintained on a 12-h-light/dark cycle. Rats were euthanized via CO_2 asphyxiation followed by thoracotomy.

Training protocol

We tested the effects of 2 weeks of exercise on GDNF protein content in the lumbar spinal cord of young (6-month-old) and old (24-month-old) animals. Two weeks of exercise was chosen as we have previously shown that this duration alters GDNF protein content in the rat skeletal muscle (McCullough et al., 2011). The 6-month-old rats were randomly divided into four groups. One group was kept as sedentary controls ($n = 12$). The remaining groups underwent different exercise protocols (swimming, voluntary running and involuntary running). The voluntary running group ($n = 6$) had continuous access to individually housed running wheels, where activity was recorded with an activity wheel monitoring system (Lafayette Instruments, Lafayette, IN, USA). The involuntary running group ($n = 5$) were placed in individual forced running wheels (Lafayette Instruments). These animals underwent five bouts of 24 min of running plus 10 min of rest, at a pace of 10 m/min (McCullough et al., 2011). Two hours of involuntary exercise was chosen to match the distance run by the voluntary running group. The swimming group ($n = 6$) had 3 rats/barrel placed in water (35 °C) and these animals swam for a total of 2 h, with bouts of rest, to match the animals of the running groups. The 24-month-old rats were randomly divided into two groups, a voluntary running group ($n = 6$), as this was the least stressful of our exercise protocols, and an age-matched sedentary control group ($n = 5$). Aged animals reached a peak running speed of only 2 m/min.

Tissue processing

In order to minimize the number of animals used for this study, we selected different regions of the spinal cord from each animal to quantify and visualize GDNF protein. The L1–L3 lumbar spinal cord region was chosen for quantification of GDNF protein content, as these motor neurons innervate the quadriceps, gluteus, adductor muscles, flexor muscles and extensor muscles, including the extensor hallucis longus, extensor digitorum longus, and the soleus (Nicolopoulous-Stournaras and Iles, 1983). Others have published GDNF protein content data from this region of the spinal cord (Tokumine et al., 2003). The lumbar spinal cord region of L4–L5 was chosen to examine localization of GDNF protein, as these motor neurons innervate the muscles of the hamstrings, adductor muscles, flexor muscles, extensor muscles including the extensor hallucis longus, extensor digitorum longus, gastrocnemius and the soleus (Nicolopoulous-Stournaras and Iles, 1983). Others have utilized immunohistochemical techniques to localize GDNF in this lumbar spinal cord region (Tokumine et al., 2003). To determine GDNF protein content, lumbar spinal cord sections (L1–L3) were removed and frozen on dry ice and samples were subsequently dipped in liquid nitrogen and smashed into a fine powder. Sample processing buffer (0.55 M NaCl, 0.02 M NaH_2PO_4, 0.08 M Na_2HPO_4, 2 mM EDTA, 0.1 mM benzethonium chloride, 2 mM benzamidine, 20 KIU/ml aprotinin, 0.5% bovine serum albumin, and 0.05% Tween-20) was added and was homogenized on ice. Samples were centrifuged for 30 min at 4 °C and supernatant was collected and stored at −80 °C.

GDNF protein quantification

GDNF protein content was measured using an enzyme-linked immunosorbent assay (ELISA) as previously described (McCullough et al., 2011). Briefly, 96-well plates were incubated overnight at room temperature in a humidified chamber with a monoclonal antibody raised against GDNF (R&D Systems, Minneapolis, MN, USA). The following day, plates were rinsed with wash buffer and blocked with phosphate-buffered saline containing 1% bovine serum albumin and 5% sucrose for 1 h at room temperature. Plates were rinsed with wash buffer and the GDNF standard (R&D Systems) or tissue supernatants were added to the wells. For each assay, a standard curve was calculated from the known GDNF standard concentration, ranging from 1000 to 2 pg/ml. Following a 2-h incubation at room temperature, the plates were washed and then incubated with biotinylated anti-GDNF secondary antibody (R&D Systems) for 2 h at room temperature. The plates were then washed and coated with β-galactosidase conjugated to streptavidin (Molecular Probes, Eugene, OR, USA) for 20 min at room temperature. The plates had a final wash and chlorophenol red-β-D-galactopyranoside (CPRG) substrate was added (in phosphate-buffered saline + bovine serum albumin) and incubated until the color had developed.

Western blot

Total protein content of the spinal cord samples was measured by a Pierce® BCA protein assay (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's specifications. Tissue samples were prepared for Western blot analysis of GDNF protein as previously described (Vianney and Spitsbergen, 2011). Briefly, protein extracts (20 μg), a protein ladder (New England BioLabs, Ipswich, MA, USA) and a loading control of α-Tubulin (Developmental Studies Hybridoma Bank, Iowa City, IA, USA) were prepared with Laemmli 2X loading buffer and loaded into a 15% polyacrylamide gel. The gel was submerged and was run in a separating buffer at different voltages followed by transfer to a polyvinylidene difluoride (PVDF; Life Technologies Corp., Carlsbad, CA, USA) membrane in tris–glycine buffer. The PVDF membrane was blocked with I-Block (Applied Biosystems, Foster City, CA, USA) followed by overnight incubation with a primary antibody against GDNF (Santa Cruz Biotechnologies, Santa Cruz, CA, USA). The following day, the membrane was washed in buffer followed by incubation with a HRP-conjugated secondary antibody (ECL; GE Healthcare Biosciences Corp., Pittsburg, PA, USA) in I-Blocking buffer. The ECL detection kit was used to detect the proteins and was visualized on BioMax XAR film (Carestream Health, Inc., Woodbridge, CT, USA). ImageJ software was used to measure the relative density of GDNF bands and values were expressed as ratios of controls.

Immunohistochemistry

Lumbar spinal cord sections (L4–L5) were fixed in 4% paraformaldehyde overnight at 4 °C and then washed in fresh phosphate-buffered saline. Tissues were embedded in optimum

cutting temperature compound (Sakura Finetek, Torrance, CA, USA) mounting medium, cut into 40-μm transverse sections on a cryotome, and thaw mounted onto Histobond® slides (VWR International, Bridgeport, NJ, USA). Slides were incubated overnight at 4 °C with primary antibodies (1:200) of rabbit anti-GDNF (Santa Cruz Biotechnology, Santa Cruz, CA, USA), (1:200) mouse anti-ChAT (Millipore, Temecula, CA), and (1:50) goat-anti GM130 (Santa Cruz Biotechnology) in phosphate-buffered saline containing 1% bovine serum albumin and 0.1% triton X-100. Slides were then incubated with secondary antibodies (1:500) of donkey anti-mouse conjugated to Alexafluor 568, donkey anti-rabbit conjugated to Alexafluor 488, and donkey anti-goat conjugated to Alexafluor 568, for 2 h at room temperature. Negative control slides had primary antibodies omitted. Slides were viewed with a Zeiss LSM 510 laser scanning confocal microscope and images were examined with the Zeiss LSM 5 Image Examiner program.

Measurement of motor neuron cell body size

Since somatic motor neurons stain positively for ChAT immunoreactivity (Wetts and Vaughn, 1996), ChAT-positive cells were measured in Lamina IX of the spinal cord from all animals. Twenty randomly selected motor neurons from the L4–L5 spinal cord levels were counted from each animal to determine cell body area. The cells that had a mid-section through the nucleus were examined. Cell body areas were determined with the Zeiss LSM 5 Image Examiner program.

Statistical analysis

All data values are reported as mean ± the standard error of the mean (SEM). GDNF protein values are expressed as pg GDNF/mg of wet tissue weight. Data were analyzed using a one-way analysis of variance (ANOVA) and Tukey's post hoc comparison to test for differences between groups. p Values $\leqslant 0.05$ were considered statistically significant.

RESULTS

Short-term exercise increases GDNF protein in the lumbar spinal cord of 6-month-old rats

Six-month-old animals underwent voluntary running, involuntary running, or swimming for 2 weeks. Animal weights were lower in the pooled exercised animals (388.8 ± 16.7 g) compared to sedentary controls (401.9 ± 24.8 g). The maximum running speed of the voluntary running group was 28 m/min, whereas the involuntary running group was maintained at 10 m/min. While the intensities were quite different between the two running groups, the average distance run per day was similar (Table 1). Two weeks of all modes of exercise significantly increased GDNF protein content in the lumbar spinal cord as compared to sedentary controls (8.6 ± 1.5 pg GDNF/mg tissue weight). Involuntary running resulted in the greatest change in GDNF protein content in the lumbar spinal cord (56.3 ± 26.4 pg GDNF/mg tissue weight), followed by swimming (25.1 ± 9.5 pg GDNF/mg tissue weight) and voluntary running (15.7 ± 1.9 pg GDNF/mg tissue weight) (Fig. 1), however there were no significant differences between the exercise groups. The involuntary running group had a 6.5-fold increase of GDNF protein content as compared to controls, followed by a 2.9-fold increase from the swimming group and a 1.8-fold increase from the voluntary running group (Table 1).

Using Western blot analysis we found that GDNF had a molecular weight of 30 kDa (Fig. 1B), which is close to the 34 kDa previously reported in human fetal spinal cord (Koo and Choi, 2001). Densitometry analysis of GDNF bands showed a 3.5-fold increase in GDNF expression for the involuntary runners, followed by a 1.7-fold increase for the swimmers and a 1.5-fold increase for the voluntary runners, which follows the same trend as our ELISA results (Table 1).

Positive immunoreactivity for GDNF was found in ChAT-positive cells, which are presumed to be motor neurons (Wetts and Vaughn, 1996), in the lumbar spinal cord from control and exercised animals (Fig. 2). We observed more vesicle-like structures containing GDNF surrounding motor neurons in exercised rats (arrows in Fig. 2B–D) compared to those from sedentary controls (Fig. 2A).

Analysis of motor neuron cell body areas revealed a significant increase following voluntary running (841.5 ± 49.6 μm^2), involuntary running (749.8 ± 29.5 μm^2), and swimming (879.9 ± 46.2 μm^2), compared to that in controls (454.8 ± 25.6 μm^2). No significant differences were observed between exercise groups. Histogram analysis of motor neuron cell body areas displayed more occurrences of the large-sized motor neurons (> 1500 μm^2) belonging to the fast motor units (Deforges et al., 2009) among the voluntary and swimming groups than the involuntary running group and controls (Fig. 3).

Short-term exercise increases GDNF protein in the lumbar spinal cord of 24-month-old rats

To determine if short-term exercise alters GDNF protein content in the spinal cord of old animals, 24-month-old rats underwent voluntary running for 2 weeks. These animals ran at a peak speed of only 2 m/min (Table 1), which is considered to be low-intensity running. Average animal weights were significantly lower in the exercised animals (355.0 ± 34.9 g) as compared to age-matched sedentary controls (415.4 ± 56.0 g). The average distance run per day by these old animals was around 1 km less than the 6-month-old runners (Table 1). Two weeks of voluntary running significantly increased GDNF protein content twofold in the lumbar spinal cord of the 24-month-old rats (87.4 ± 4.6 pg GDNF/mg Tissues) as compared to age-matched sedentary controls (54.4 ± 9.3 pg GDNF/mg Tissue) (Fig. 4a). GDNF protein levels in the spinal cord were significantly higher among the 24-month-old controls than the 6-month-old controls.

Western blot analysis also confirmed that 2 weeks of exercise increased GDNF protein expression twofold in the lumbar spinal cord of old rats as compared to age-matched sedentary controls (Fig. 4b). Interestingly, the molecular weight of GDNF appeared to be around 41 kDa in old rats compared to 30 kDa in young adult rats.

Immunoreactivity for GDNF followed a similar pattern to that observed in 6-month-old rats, where we found more vesicle-like structures containing GDNF outside of ChAT-positive motor neurons in exercised animals compared to sedentary controls (Fig. 5A, B). Antibody staining for colocalization of GDNF to vesicle-like

Table 1. Low-intensity, forced running (10 m/min) elicited the greatest increase in GDNF protein content in the spinal cord compared to other modalities of exercise. Changes in GDNF protein content in the spinal cord, as detected by Western blot, show similar trends as those measured via ELISA. Old rats (24-month-old) ran a shorter distance and at a lower intensity than young (6-month-old) rats.

Age of animals (months)	Duration and type of exercise	Fold change of GDNF protein content (via ELISA) from controls[a]	Fold change of GDNF protein content (via Western blot) from controls	Maximum intensity of exercise (m/min)	Distance run/ day (m)
6	2 weeks voluntary running	2 ↑	1.5 ↑	28	1434.9 ± 77.4
6	2 weeks involuntary running	6.5 ↑	3.5 ↑	10	1200
6	2 weeks swimming	3 ↑	1.7 ↑	N/A	N/A
24	2 weeks voluntary running	2 ↑	2 ↑	2	132.3 ± 86.6

[a] Involuntary running elicits the largest increase of GDNF protein content in the rat spinal cord as measured via ELISA.

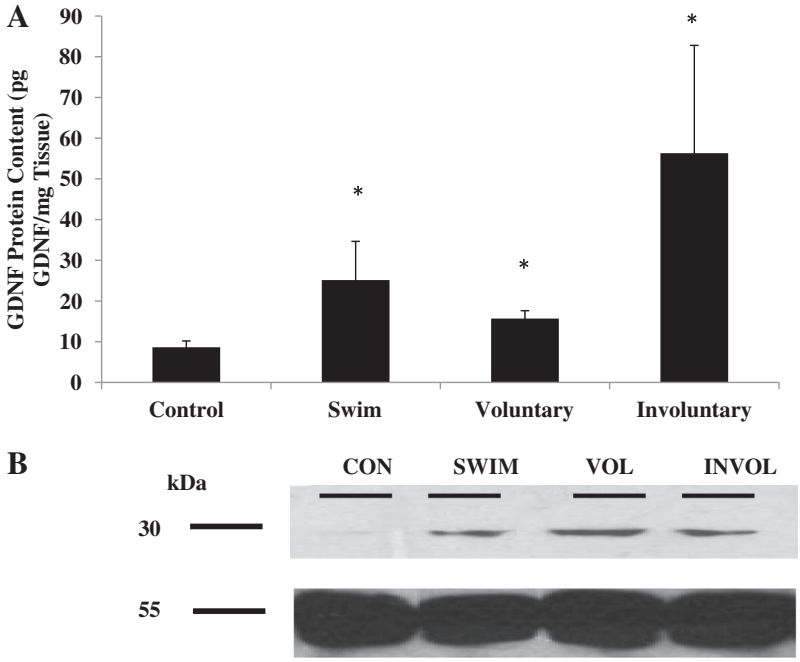

Fig. 1. GDNF protein content was increased in the spinal cord after 2 weeks of exercise in 6-month-old rats. The lumbar spinal cord (L1–L3) was removed from control and exercised 6-month-old animals. (A) Tissues were processed for GDNF protein content using an ELISA. A significant increase in GDNF protein content was detected in the spinal cord of animals that had undergone 2 weeks of voluntary running, involuntary running and swimming as compared to sedentary control animals. Values are displayed as mean ± SEM. Asterisk (∗) indicates significance ($p \leqslant 0.05$) from controls. (B) Tissues were processed for Western blot to determine GDNF protein content (top) and a loading control of α-tubulin (bottom). An increase in GDNF protein content was detected in the spinal cord of animals that had undergone 2 weeks of involuntary running, followed by swimming and then voluntary running as compared to controls.

structures was confirmed with the GM130 antibody (Fig. 5C).

ChAT-positive motor neuron cell body area in 24-month-old animals was significantly greater following 2 weeks of exercise (618.8 ± 31.5 μm^2) compared to that in age-matched sedentary controls (387.1 ± 18.0 μm^2). The ChAT-positive motor neuron cell body area from the 24-month-old sedentary controls were significantly smaller than the 6-month-old sedentary controls ($p = 0.02$). Furthermore, the ChAT-positive motor neuron cell body area from the 24-month-old voluntary runners were significantly smaller than the 6-month-old voluntary runners ($p = 0.003$). Again, histogram analysis of ChAT-positive motor neuron cell body areas displayed a higher frequency of the large-sized motor neurons (> 1500 μm^2) following 2 weeks of voluntary running as compared to controls (Fig. 6).

DISCUSSION

While other investigators have found that exercise increases neurotrophin levels in the spinal cord (Gomez-Pinilla et al.,

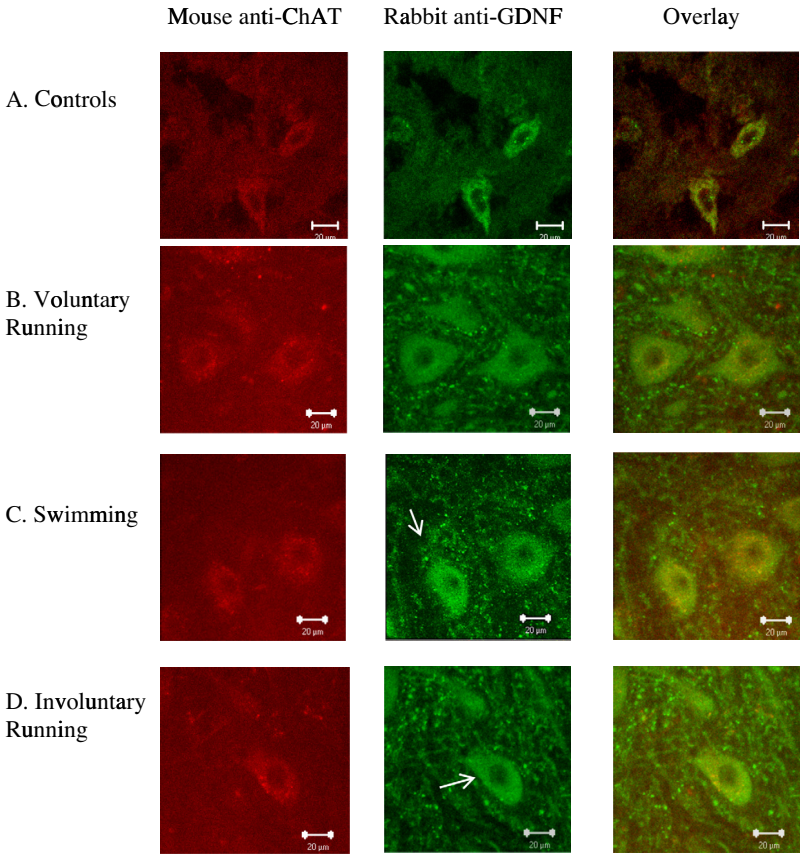

Fig. 2. Exercise increased motor neuron size and vesicle-like structures of GDNF in 6-month-old rat spinal cord. Representative lumbar spinal cord sections from a 6-month-old sedentary control animal (A), voluntary exercised animal (B), swimming exercised animal (C) and an involuntary exercised animal (D). Spinal cord sections were immunolabeled with primary antibodies against ChAT (red) and GDNF (green). ChAT immunoreactivity is co-localized with GDNF immunoreactivity in the lumbar spinal cord. Exercised animals appeared to have more vesicle-like structures containing GDNF that surround the motor neurons (indicated by arrows), as compared to controls. The scale bar represents 20 μm.

2001, 2002; Dupont-Versteegden et al., 2004), few studies have examined the effects of exercise on spinal cord GDNF levels. The present study was designed to determine if short-term exercise (2 weeks) would alter GDNF protein content in the spinal cord of young and old animals. In young rats, involuntary running resulted in the greatest fold-change in GDNF protein content in the spinal cord, followed by swimming and voluntary running, however these levels were not statistically different. Interestingly, both young and old voluntary runners displayed the same fold-change in GDNF protein content as compared to their age-matched controls. In the exercise groups, ChAT-positive motor neuron cell body area doubled in size compared to that from age-matched sedentary controls.

Motor neuron size increases at the same time as GDNF levels following short-term exercise

In animal models of aging, there is selective atrophy of large-sized motor neurons and a decrease in the total number of motor neurons that innervate hindlimb muscles (Hashizume and Kanda, 1990; Hirofuji et al., 2000). Our results confirm that motor neuron cell body size decreases with advancing age in sedentary animals. Mature motor neurons obtain trophic support from various types of cells, including Schwann cells, skeletal muscle cells and other neurons (Nishi, 1994; Oppenheim, 1996), and may resist death by increasing production of neurotrophic factors in these tissues. While there may not be a direct correlation to the increase in neurotrophic factor content with the decrease in motor neuron size observed with aging, our observations of increased neurotrophic levels with advancing age may suggest a steady increase in response to motor neuron loss with senescence. Acute effects of exercise were also found to increase production of neurotrophic factors. Recent studies have demonstrated links between beneficial effects of exercise, changes in neurotrophic factor levels and neuronal plasticity. Our observations of increased neurotrophic factor expression in the lumbar spinal cord following short-term exercise coupled with the increase in motor neuron size suggest acute bouts of exercise as a possible mechanism to protect motor neurons from undergoing atrophy with senescence.

Low-intensity exercise is a potent stimulus for enhancing neurotrophic factor levels

While some studies report that voluntary exercise increases mRNA and protein levels for neurotrophic

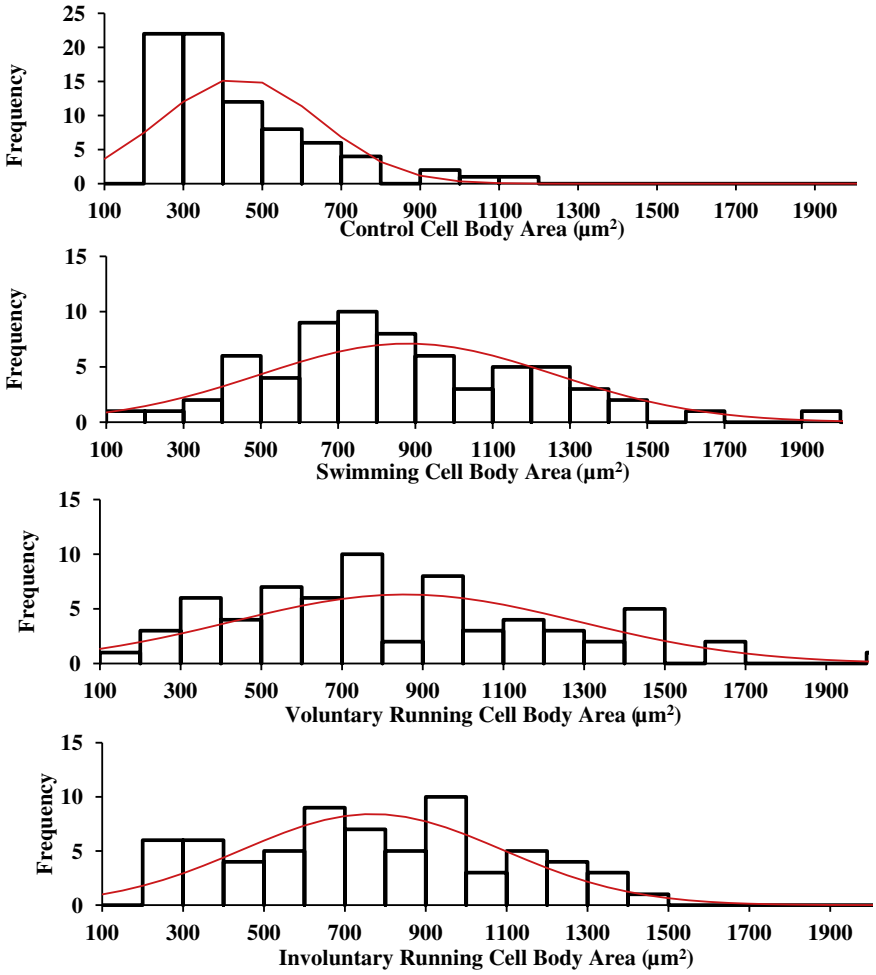

Fig. 3. Histogram analysis of ChAT-positive motor neuron cell body area of 6-month-old rats. Following 2 weeks of exercise, the voluntary running group and swimming group displayed a higher frequency of cells $>1500\ \mu m^2$ as compared to the involuntary running group and controls.

factors in the spinal cord (Macias et al., 2002; Skup et al., 2002; Ying et al., 2003; Ferraiuolo et al., 2009), others show decreasing levels of neurotrophic factors in the spinal cord following exercise (Engesser-Cesar et al., 2007; Siamilis et al., 2009). One possible contributing factor to these discrepancies may be due to variations in intensity and duration of exercise. BDNF and neurotrophin-3 mRNA and protein levels in the lumbar spinal cord are known to increase following short-term exercise (Neeper et al., 1996; Gomez-Pinilla et al., 2001; Molteni et al., 2002). Moderate-intensity exercise (13 m/min) increases BDNF, but high intensity exercise decreases BDNF levels in the brain (Aguiar et al., 2007), suggesting that low to moderate intensities of exercise may be a more potent stimulus for increasing neurotrophic factor levels. Our results lend support to this idea, where we find that our moderate-intensity involuntary running protocol yielded the greatest change in GDNF protein content of all exercise regimens examined. Together, these results may suggest that short-term, moderate-intensity exercise programs may be a better stimulus for enhancing neurotrophic factor content in the spinal cord.

Punctate immunoreactivity for GDNF is altered with exercise and age

A punctate staining pattern for GDNF has been found in neuronal cell bodies, dendrites and axons (Kawamoto et al., 2000). In cultured neuroendocrine cells the staining pattern for GDNF appears to be localized to vesicle-like structures (Lonka-Nevalaita et al., 2010). Moreover, in axons of dorsal root ganglion neurons GDNF is present in dense-core vesicles (Ohta et al., 2001) and in rat primary cortical and hippocampal neurons, the immunostaining pattern of GDNF appears in vesicle-like structures at the tips of neurites, where the authors suggest transportation of GDNF to the cell periphery (Lonka-Nevalaita et al., 2010). Within the spinal cord, we observed vesicle-like staining for GDNF that was confirmed with the GM130 antibody, where our results suggest that exercise increases the incidence of GDNF-positive immunoreactivity. These observations are in accordance with our ELISA values, where GDNF protein content is increased following exercise in both young and old animals as well as increased with advancing age in control animals.

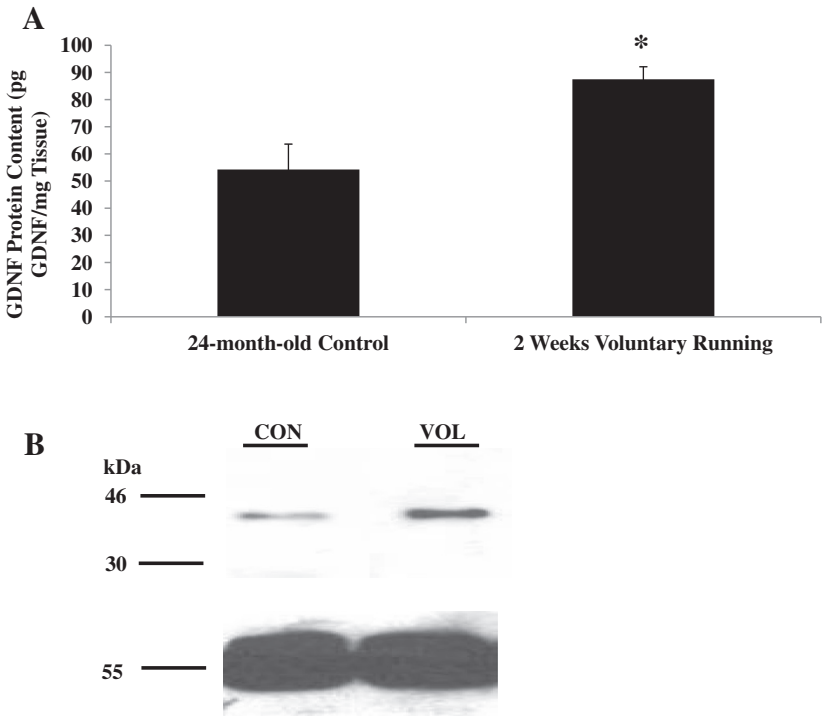

Fig. 4. GDNF protein content was increased in the spinal cord after 2 weeks of exercise in 24-month-old rats. The lumbar spinal cord (L1–L3) was removed from control and exercised 24-month-old animals. (A) Tissues were processed for determination of GDNF protein content using an ELISA. A significant increase in GDNF protein content was detected in the lumbar spinal cord of animals that had undergone 2 weeks of voluntary running as compared to age-matched sedentary controls. Values are displayed as mean ± SEM. Asterisk (∗) indicates significance ($p \leqslant 0.05$) from controls. (B) Tissues were processed for Western blot to determine GDNF protein content (top) and a loading control of α-tubulin (bottom). An increase in GDNF protein content was detected in the spinal cord of animals that had undergone 2 weeks of voluntary running as compared to controls.

Molecular weight of GDNF changes with age

The reported molecular weight of GDNF varies in the literature depending on the cells/tissues examined. Lin et al. (1994) describe GDNF from cultured dopaminergic cells as having a molecular weight of 33–45 kDa in non-reduced gels and 15–21 kDa from reduced gels. GDNF protein size was reported to be 24 kDa in cultured primary fibroblasts (Blesch and Tuszynski, 2001), 30 kDa in cultured neuroblastoma cells (Larsen et al., 2006) and 34 kDa in the 18-week-old human fetal spinal cord (Koo and Choi, 2001). We found the molecular weight of GDNF in the rat lumbar spinal cord to be 30 kDa in young animals and 41 kDa in old animals. Similarly, several nerve growth factor (NGF) isoforms have been reported in various whole tissues of both humans and animals (reviewed by Al-Shawi et al., 2007). One explanation for the variations in molecular weights may be due to modifications of the prodomain regions of the protein. Pro-neurotrophins, which are the precursor forms of neurotrophins, are synthesized and then cleaved by furin and other proteases to produce mature neurotrophins (Lee et al., 2001). Mature NGF and BDNF induce neuronal survival, differentiation and synaptic modulation (Huang and Reichardt, 2001). Results of other studies suggest that the precursor of NGF may be either neurotoxic (Ibanez, 2002) or significantly less neurotrophic than the mature form of NGF (Fahnestock et al., 2004). Pro-neurotrophins, such as pro-BDNF and pro-NGF, induce cell death by activating an apoptotic cascade via binding to cell death complexes involving sortilin and p75 receptors (Lee et al., 2001; Nykjaer et al., 2004). Moreover, pro-NGF has been found to be upregulated with aging and disease. Pro-NGF is increased in the superior cervical ganglia of old rats (Bierl and Isaacson, 2007), the parietal cortex of patients with Alzheimer's disease (Peng et al., 2004) and in spinal cord oligodendrocytes from a murine model of spinal cord injury (Beattie et al., 2002). Our observation of increasing molecular weight for GDNF with advancing age may suggest a similar phenomenon is occurring in the spinal cord of rat, possibly demonstrating increased expression of a higher molecular weight pro-form of GDNF in the older animals. It has been shown that pro-NGF is secreted by reactive astrocytes, and may affect motor neuron survival (Domeniconi et al., 2007). The spinal cord samples processed in the current study would contain motor neurons, interneurons and glial cells, which could account for the pro-GDNF, if it is associated with glial cells. While it is still unknown if the pro-form of GDNF activates similar apoptotic pathways as NGF, it is known that post-translational modifications of GDNF are due to prohormone convertase that cleaves five consensus sites giving rise to four different peptide forms of processed GDNF (Immonen et al., 2008; Oh-hashi et al., 2009). Future studies are warranted to determine if pro-GDNF is less neuroprotective or neurotoxic and how aging affects its expression.

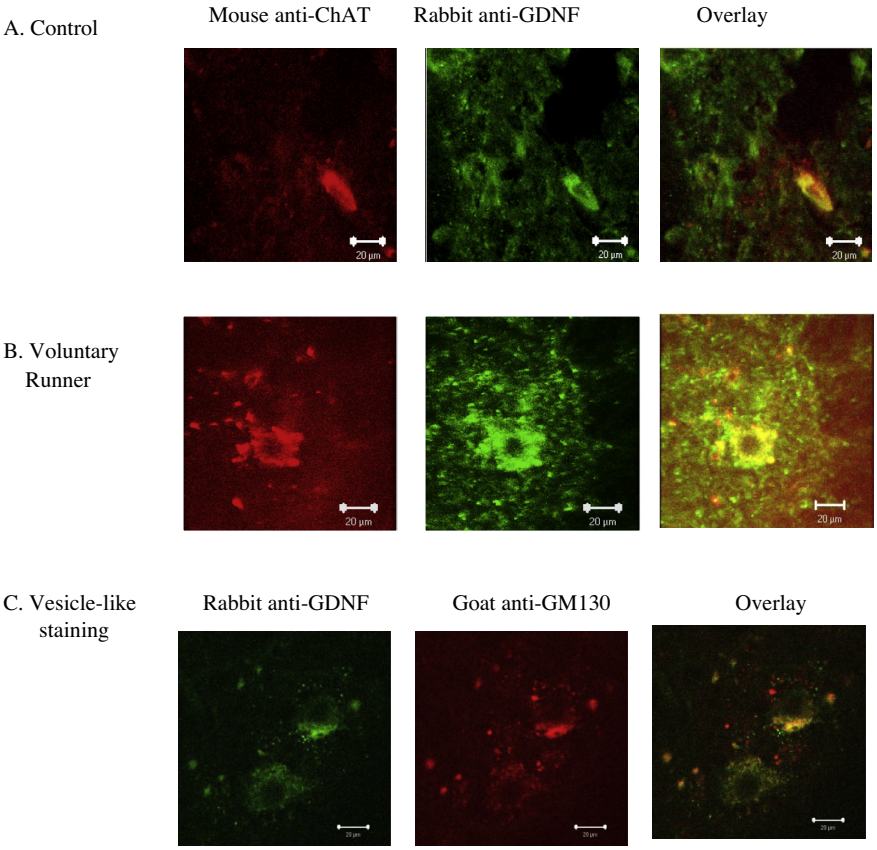

Fig. 5. GDNF immunoreactivity and motor neuron cell body size increased with exercise in 24-month-old rats. Representative lumbar spinal cord sections from a 24-month-old control animal (A) and a 2-week voluntary exercised animal (B). Spinal cord sections were immunolabeled with primary antibodies against ChAT (red) and GDNF (green). Exercised animals appeared to have more vesicle-like structures containing GDNF that surrounded the motor neurons (arrows in B) as compared to controls (arrows in A). GDNF immunoreactivity was also colocalized to the GM130 antibody for vesicle-like structures (C). Scale bar = 20 μm.

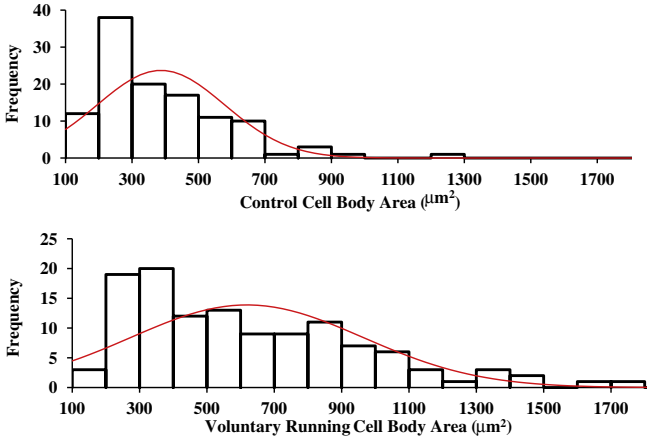

Fig. 6. Exercise increased ChAT-positive motor neuron cell body area of 24-month-old rats. Histogram analysis of ChAT-positive motor neuron cell body area. Following 2 weeks of exercise, voluntary runners displayed a higher frequency of cells >1500 μm² as compared to 24-month-old sedentary controls.

GDNF transport following exercise

GDNF is produced by motor neurons, oligodendrocytes and Schwann cells in the spinal cord (Henderson et al., 1994; Yamamoto et al., 1996; Russell et al., 2000; Rind and von Bartheld, 2002) as well as by skeletal muscles (Yamamoto et al., 1996). Both anterograde and retrograde transport between neurons and target tissues

have been demonstrated for GDNF (Russell et al., 2000; Rind and von Bartheld, 2002). While our results did not determine which cells are producing the GDNF protein observed in the spinal cord, we have previously shown that GDNF protein is increased in the skeletal muscle following short term exercise (Wehrwein et al., 2002; McCullough et al., 2011), which could be transported back to the spinal cord, resulting in the elevated levels observed in the current study. It has been shown that skeletal muscle derived GDNF has more potent effects for the neuromuscular system than that supplied by anterograde transport (Li et al., 2007), thus elevated retrograde transport of GDNF from the muscle following exercise may represent an important stimulus for enhanced plasticity.

Exercise stressors

There is evidence that stress is unlikely to be the critical factor underlying the differential effects of voluntary and forced running (Leasure and Jones, 2008). Forced running acutely elevates corticosterone levels, the rodent stress hormone, more than voluntary running (Ploughman et al., 2005, 2007), however these levels return to baseline within a few hours after exercise (Stranahan et al., 2006; Ploughman et al., 2007) and after several weeks of exercise these levels are no longer elevated (Fediuc et al., 2006). Stress is also known to activate microglia (Nair and Bonneau, 2006; Sugama et al., 2007), however neither forced nor voluntary running enhances microglial activity in the brain (Leasure and Jones, 2008). These observations may suggest that the exercise-induced changes in neurotrophic factor expression observed in the current study are not likely to be dependent on a stress response.

CONCLUSION

In conclusion, the results demonstrate that short-term exercise increases GDNF protein content, GDNF immunolabeling and motor neuron size in the spinal cord of young and old animals. These results are consistent with our hypothesis that the neural protection/neural plasticity caused by exercise may be driven, in part, by enhanced GDNF production. In addition, there may be a relationship between the intensity of exercise and the amount of GDNF protein produced, where a low-intensity exercise protocol yields the greatest increase in GDNF protein content. We believe that exercise has the advantage of enhancing neurotrophic factor levels by physiological means using intrinsic mechanisms in the spinal cord rather than attempting to increase neurotrophic factor levels via exogenous administration where all the physiological implications are not well understood.

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APPENDIX E

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REVIEW

Exercise-dependent regulation of glial cell line-derived neurotrophic factor (GDNF) expression in skeletal muscle and its importance for the neuromuscular system

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Abstract **The focus of this review is to highlight the importance of glial cell line-derived neurotrophic factor (GDNF) for the motor nervous system. GDNF is the most potent survival factor for motor neurons, where it enhances maintenance and survival of both developing and mature motor neurons *in vivo* and *in vitro*. GDNF aids in neuromuscular junction formation, maintenance, and plasticity, where skeletal muscle-derived GDNF may be responsible for this phenomenon. Increased levels of physical activity can increase GDNF protein levels in skeletal muscle, where alterations in acetylcholine and acetylcholine receptor activation may be involved in regulation of these changes observed. With inactivity and disuse, GDNF expression shows different patterns of regulation in the central and peripheral nervous systems. Due to its potent effects for motor neurons, GDNF is being extensively studied in neuromuscular diseases.**

Keywords glial cell line-derived neurotrophic factor, neuromuscular junction, motor neurons, skeletal muscle

Neurotrophic factors

Neurotrophic factors are a family of small peptides that have been identified as important factors for the development, survival, plasticity, and function of neurons both in the central nervous system (CNS) and peripheral nervous systems (PNS) (Henderson et al., 1994; Allen and Dawbarn, 2006). The basic model of target-derived neurotrophic factors proposes that a target tissue produces a molecular signal that is recognized by innervating neurons and is capable of enhancing the growth and survivability of those neurons during development (Purves et al., 1988). Neurotrophic factors can be classified in several subgroups including the neurotrophin family, which consist of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4), the neurocytokine family, which includes ciliary neurotrophic factor, leukemia inhibitory factor (LIF) and interleukins, and the glial cell line-derived neurotrophic factor (GDNF) family, which includes GDNF, neurturin (NTRN), artermin (ARTN), and persephin (PSPN).

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GDNF characterization and signaling

GDNF was first purified from B49 glial cells and was identified as a potent survival factor for dopaminergic neurons in the CNS (Lin et al., 1993; Schatz et al., 1999). Later, GDNF was reported to be a trophic factor for other populations of neurons in the PNS including spinal motor neurons (Henderson et al., 1994; Niles et al., 2004; Caumont et al., 2006; Sharma, 2006), where it has been identified as one of the most potent neurotrophic factors for motor neurons to date (Henderson et al., 1994; Oppenheim et al., 1995; Trupp et al., 1995; Yan et al., 1995; Nguyen et al., 1998). GDNF is synthesized as an inactive 211 amino acid precursor form, pre-proGDNF (Lin et al., 1993; Airaksinen and Saarma, 2002). The mature forms of GDNF are expressed in two splice variants, $GDNF_{633}$ and $GDNF_{555}$, that are disulfide-bonded homodimers of two 134-residue long glycosylated polypeptide chains and are obtained after proteolytic cleavage during secretion (Suter-Crazzolara and Unsicker, 1994; Springer et al., 1995; Airaksinen and Saarma, 2002). The mature forms are thought to be released in a biologically

active state and are able to initiate signaling with tyrosine kinase receptors (Lee et al., 2001). All the members of the GDNF family induce signaling through binding with high affinity to the GDNF family receptor α (GFR-α1-4), which are anchored to the plasma membrane by a glycosyl-phosphatidylinositol (GPI) anchor. Specifically, GDNF binds to GFR-α1, NTRN binds to GFR-α2, ARTN binds to GFR-α3, and PSPN binds to GFR-α4, however there is cross-talk among the ligands to other receptors in which they bind with lower affinity. The GDNF-GFR-α1-GPI complex interacts with two RET molecules, which are single-pass transmembrane proteins, thus initiating homodimerization and tyrosine autophosphorylation (Airaksinen and Saarma, 2002). This in turn triggers downstream signaling cascades including phosphatidylinositol 3-kinase, extracellular regulated kinase, and mitogen-activated protein kinase (Soler et al., 1999). These same pathways have been linked to neuronal survival, synaptic plasticity, neurite outgrowth, and the enhancement of neurotransmission (Kaplan and Miller, 2000; Yang et al., 2001).

GDNF's role for the neuromuscular system

Developing motor neurons

GDNF was found to be 75-, 650-, and 2500-fold more potent in preventing programmed cell death in cultured motor neurons than BDNF, rat ciliary neurotrophic factor (CNTF) and human cholinergic differentiation factor leukemia inhibitory factor, respectively. In this same study, administration of GDNF prevented death of nearly all facial motor neurons in neonatal rats and was the only trophic factor capable of averting axotomy-induced atrophy *in vivo* (Henderson et al., 1994). GDNF treatment was shown to prevent death and atrophy of nearly 100% of axotomy-induced motor neurons in chick embryos (Oppenheim et al., 1995) and facial motor neurons in neonatal rats as compared to axotomized controls in which less than 6% of motor neurons remained (Yan et al., 1995). In addition, overexpression of GDNF in developing muscle showed an increase in survival of nearly all motor neuron populations (spinal and cranial) during naturally occurring cell death compared to GDNF-deficient embryos (Oppenheim et al., 2000), where the survival of motor neurons during programmed cell death depends on GDNF secreted specifically by skeletal muscle (Angka et al., 2008). Excessive cell death was observed in double mutant embryonic mice lacking both skeletal muscle and their trophic factors. In contrast, these double mutants treated with GDNF had the same level of motor neuron survival as that in wild type mice (Angka et al., 2008). Although GDNF has been characterized as a neurotrophic factor for alpha-motor neurons (Henderson et al., 1994), differential effects on motor neuron subtypes have been observed during development. Recently, it has been reported that gamma-motor neurons express higher levels of GDNF receptor (GFRα-1) compared to alpha-motor neurons in the early postnatal period, suggesting that early gamma-motor neurons depend on muscle spindle-derived GDNF (Shneider et al., 2009; Kanning et al., 2010). GDNF also plays a chemoattractant role in axon pathfinding during development. Studies have shown that GDNF acts with ephrinA/ephrinA receptor, the complex that induces motor neuron repulsion, to ensure the correct connection of motor axons in limbs of animal models (Kramer et al., 2006; Dudanova et al., 2010). These studies indicate that developing and axotomized motor neurons depend on access to GDNF for survival, and possibly from muscle-derived GDNF rather than centrally-derived GDNF.

Mature motor neurons

GDNF availability has been found to be crucial for the maintenance of mature adult motor neurons. Disease and nerve injury studies have revealed the trophic importance of GDNF to motor neurons in adults. Administration of GDNF prevented neuronal loss and maintained motor function in a mouse model of Huntington's disease (Ebert et al., 2010). Also, GDNF synthesized from healthy mouse femur bone marrow was sufficient to slow motor neuron degeneration in transgenic mice with muscle *deficient/osteocondrodystrophy* mutation (*mdf/ocd*: Pastor et al., 2011).

GDNF mRNA was shown to be upregulated at the distal part of injured nerves, skeletal muscle, and Schwann cells after transection (Naveilhan et al., 1997; Frostick et al., 1998; Lie and Weis, 1998). The primary GDNF receptor, GFR-α1, was also increased at the distal part of the nerve after nerve lesion (Frostick et al., 1998). This elevation of GDNF mRNA may suggest an immediate trophic need by the damaged nerve. More importantly, GDNF mRNA remained elevated for five or six weeks; in contrast with members of the neurotrophin family whose upregulation existed for only two weeks (Frostick et al., 1998; Michalski et al., 2008). From this, it can be suggested that adult motor neurons depend primarily on GDNF as their trophic factor for survival and that GDNF is critical for the maintenance of the neuromuscular system.

Neuromuscular systems

The presence of GDNF in skeletal muscle at the neuromuscular junction (NMJ) suggests a target-derived action on motor neurons, in which GDNF is retrogradely transported by axons to the target neuron's cell body, through a receptor-mediated process (Nguyen et al., 1998). GDNF has also been shown to be secreted by astrocytes of the CNS, exerting its effects on the PNS by anterograde transport (Zhao et al., 2004). GDNF appears to increase acetylcholine receptor (AChR) density in the absence of innervation, indicating an autocrine action, since both the ligand and its receptor are

expressed in skeletal muscle (Yang and Nelson, 2004). Studies examining transgenic mice overexpressing GDNF in skeletal muscle (*myo*-GDNF) and transgenic mice overexpressing GDNF in astrocytes (*GFAP-GDNF*) showed that the classical retrograde transport may have more physiologic effects for treatment of amyotrophic lateral sclerosis (ALS) compared to the anterograde direction (Li et al., 2007).

GDNF has been shown to be critical for NMJ formation, maintenance, and plasticity both *in vivo* and *in vitro*. Studies have shown that during synaptic formation, the number of axons innervating skeletal muscle depends on the concentration of GDNF protein available (Nguyen et al., 1998). Elevated GDNF expression causes hyperinnervation at the NMJ of transgenic *myo*-GDNF mice, whereas *myo*-neurotrophin-3 (NT-3) mice and *myo*-neurotrophin-4 (NT-4) mice have no effect (Nguyen et al., 1998). Adult mice overexpressing GDNF under myosin light chain 1(MLC1) promoter were shown to maintain hyperinnervation of skeletal muscles, showing a 70% increase in the number of endplates when compared to wild-type animals (Zwick et al., 2001). Multiple innervations of endplates is also observed when continuous administration of exogenous GDNF is initiated after birth into adulthood (Keller-Peck et al., 2001). In addition to GDNF-induced hyperinnervation, continuous treatment with GDNF in *Xenopus* nerve-muscle coculture enhanced axonal growth by increasing the length of neurites in motor neurons (Wang et al., 2002). These results suggest that GDNF plays a significant role in synaptic maintenance and remodeling of the NMJ into adulthood.

GDNF acts on both presynaptic and postsynaptic apparatus during synaptic transmission. GDNF increases spontaneous neurotransmitter release in neonatal mice. Of the neurotrophic factors examined: GDNF, BDNF, NT-4, LIF, insulin-like growth factor (IGF), and IGF-2, GDNF was the only one to increase frequency of spontaneous neurotransmitter release by twofold as compared to the other neurotrophic factors (Ribchester et al., 1998). Other studies show that GDNF not only potentiates spontaneous synaptic transmission, but also enhances evoked transmitter release. Treatment with GDNF increased the spontaneous frequency of neurotransmitter release 5-fold and the evoked neurotransmitter release twofold in *Xenopus* nerve-muscle cocultures (Wang et al., 2001). GDNF elicits a small increase in quantal size without affecting the average rise and decay times of synaptic currents (Wang et al., 2002). Exogenous application of other neurotrophic factors, such as BDNF, can increase the frequency of spontaneous synaptic currents (Stoop and Poo, 1996). It was found that GDNF induces activity in the presynaptic terminal by enhancing expression of frequenin, an N-type calcium binding protein, hence facilitating calcium influx into the nerve terminal (Wang et al., 2001). Again, the action of GDNF on frequenin and calcium influx was specific to GDNF because other molecules such as NT-3 and NT-4 had no effect (Wang et al., 2001). In the postsynaptic cell, GDNF was shown to increase the size of AChR aggregates (Wang et al., 2002) as well as the insertion rate of these receptors (Yang and Nelson, 2004). Taken together, it can be suggested that GDNF promotes axon branching, enhances synaptic formation, maintains synaptic connection, and modulates mammalian NMJ through both presynaptic and postsynaptic actions.

GDNF expression following exercise

A role for neurotrophic factors has been proposed as contributing to exercise-induced changes in the nervous system (Wehrwein et al., 2002; Adlard and Cotman, 2004; McCullough et al., 2011). Neurotrophic factors, such as BDNF, IGF-1, and vascular endothelial growth factor (Wu et al., 2008; Trejo et al., 2001; Fabel et al., 2003), have also been suggested to play a role in exercise-mediated neuroprotective effects, where levels of expression of BDNF and NT-3 are increased in the spinal cord and skeletal muscle following both involuntary and voluntary exercise (Gómez-Pinilla et al., 2001, 2002). A relationship between neurotrophic factors and physical activity has been demonstrated with concentrations of NT-4 in skeletal muscle changing in proportion to the intensity of exercise (Funakoshi et al., 1995). We have previously shown that 4 weeks of treadmill training and 2 weeks of forced wheel running can increase GDNF protein content in rat skeletal muscle, while decreases in GDNF protein content were observed with hindlimb suspension (Wehrwein et al., 2002; McCullough et al., 2011) (Table 1). Following nerve injury, exercise increases GDNF mRNA in rat skeletal muscle (Dupont-Versteegden et al., 2004). Injection of botulinum toxin type A into skeletal muscle has been shown to block the beneficial effects of voluntary exercise on BDNF expression, suggesting that ongoing neuromuscular activity is important in regulating neurotrophic factor expression in skeletal muscle (Gómez-Pinilla et al., 2002).

Exercise-induced GDNF expression following spinal cord injury

Spinal cord injuries interfere with an individual's daily activity. However, no standard treatment for spinal cord injury (SCI) has been implemented as it requires various treatments according to the site of injury. Neurotrophic factors, such as BDNF and GDNF, have been regarded as potential candidates for SCI treatment (Cote et al., 2011). Particularly, levels of GDNF protein and its mRNA are upregulated after SCI, indicating a trophic role to the damaged spinal cord (Hashimoto et al., 2005; Zhou et al., 2008). *In vivo* and *in vitro* administration of exogenous GDNF have shown promising results. GDNF enhances axonal regeneration and myelination *in vivo* following SCI whereas in a coculture of Schwann cells and neurons of the dorsal root ganglia, GDNF increases myelin sheath cells

Table 1 Regulation of GDNF protein production

Factor	Effect	Site	References
Walk training	–	EDL	McCullough et al., 2011; Wehrwein et al. 2002
	+	SOL	
Stretch	none	EDL	McCullough et al., 2011
	+–	SOL	
Electrical stimulation	–	EDL	McCullough et al., 2011
	+	SOL	
Hindlimb suspension	–	SOL	Wehrwein et al. 2002
	+	Pectoralis major	
Cholinergic neurons	–	Skeletal muscle cells in culture	Vianney and Spitsbergen, 2011
nAChR activation	–	Skeletal muscle cells in culture	Vianney and Spitsbergen, 2011
Spinal cord injury	+	Skeletal muscle	Lie and Weis, 1998; Suzuki et al., 1988a; Trupp et al., 1995; Naveilhan et al., 1997
	+	Spinal cord	Hashimoto et al., 2005
Spinal cord injury and exercise	+	SOL	Dupont-Versteegden et al., 2004
	+	Spinal cord	Cote et al., 2011
Aging	–	Skeletal muscle	Nagano and Suzuki, 2003

(Zhang et al., 2009). In SCI animal models, GDNF protein was shown to increase 14-fold in animals subjected to exercise as compared to sedentary control groups (Dupont-Versteegden et al., 2004), suggesting that the release of GDNF may be exercise-dependent. Interestingly, the increased levels of GDNF in exercised animals correlate with SCI recovery (Cote et al., 2011) supporting that exercise training can be beneficial for restoration of injured spinal cord via increasing release of GDNF.

Potential mechanisms

Possible signaling mechanisms that may be impacting GDNF expression following exercise, include altered neurotransmitter release, activation of AChRs, electrical stimulation and stretch. A recent study has found that addition of cholinergic neurons inhibits GDNF secretion by skeletal muscle cells (Vianney and Spitsbergen, 2011) (Table 1). Total GDNF (in both cell culture medium and inside cells) was decreased 36% when cells were cocultured as compared to muscle cell cultures. Earlier reports show similarities to this finding where high amounts of GDNF are found after skeletal muscle denervation, where it may be acting as a first responder to provide trophic support for reinnervating neurons (Lie and Weis, 1998; Suzuki et al., 1998a, b). Together, these results suggest that upon synaptic contact, motor neurons may inhibit production of GDNF in skeletal muscle and only allow the amount of GDNF to be released that is needed to maintain normal structure and function of the neuron. Electrical stimulation at a low frequency (0.1 Hz) has been shown to alter GDNF protein content in rat skeletal muscle and pretreatment with α-bungarotoxin blocks those effects (McCullough et al., 2011) (Table 1). Addition of α-bungarotoxin to nerve-muscle cocultures reverses the inhibitory effects of cholinergic neurons on skeletal muscles, suggesting that activation of nicotinic acetylcholine receptors (nAChRs) may be decreasing GDNF expression (Vianney and Spitsbergen, 2011) (Table 1). Others have found that neurotransmitters from sympathetic neurons can alter the level of secretion of NGF in vascular and bladder smooth muscle cells (Spitsbergen et al., 1995; Clemow et al., 1999). Together, these results suggest that ACh is acting on nAChRs to regulate neurotrophic factor expression in muscle cells, where increased nAChR activation leads to a decrease in neurotrophic factor expression. This inhibitory effect of neurotransmitters on GDNF expression may provide one explanation for elevated GDNF mRNA levels following denervated skeletal muscle from individuals suffering from ALS (Lie and Weis, 1998) and for enhanced survival of developing motor neurons following blockade of nAChRs (Oppenheim et al., 2000). Furthermore, a direct relationship between stretching of muscle and the amount of neurotransmitter released may be another reason for changes in neurotrophic factor expression following exercise (Chen and Grinnell, 1997). Stretching muscles *in vitro* was shown to increase GDNF protein content after only 4 h, where blockade of nAChRs followed by stretch leads to a large increase in GDNF protein content (McCullough et al., 2011). Thus, mechanical activity of skeletal muscles may be an important mediator to increase neurotrophic factor expression with exercise. Different modalities of exercise have been found to alter the nerve terminal area, such as with low-intensity exercise (Andonian and Fahim, 1987; McCullough et al., 2011) and with low-frequency electrostimulation (Mussini and Marchioro, 1991). It has been suggested that there is a difference in the amount of storage and release of ACh in skeletal muscles upon exercise demands (Dorlöchter et al., 1991; Deschenes et al., 1993; Wood and Slater, 1997; Reid et al., 1999). Further investigation needs to be done to

determine how exercise increases GDNF in the neuromuscular system.

GDNF's role with aging

Deficiency of GDNF has been reported to accelerate age-related problems (Boger et al., 2006). Aged individuals show a decline of GDNF in substantia nigra that is associated with motor dysfunction (Salvatore et al., 2004). Because GDNF knockout mice do not survive due to undeveloped kidneys (Moore et al., 1996), researchers have been using heterozygous mice to study consequences of decreased GDNF expression. In a study conducted by Borger et al., a partial deletion of the *GDNF gene* in heterozygous mice caused an age-related motor dysfunction and a significant decrease in tyrosine hydroxylase (TH) expression compared to that in wild type mice (Boger et al., 2006). It had been reported that administration of GDNF improved motor function recovery in aged rat. This effect was shown to be mediated through the increase of TH in striatum and substantia nigra, which catalyzes the dopamine synthesis from their precursors, hence dopaminergic neurons activity (Salvatore et al., 2004). Thus GDNF may be important in maintaining motor function, possibly through TH enzyme expression. Furthermore, during aging, it has been observed that nerves start producing growth-associated protein 43 (GAP43) suggesting that aging nerves may be switching from transmitting function to growth mode in order to promote survival and reinnervation, which was reported in axotomized nerves (Fu and Gordon, 1997; Ulfhake et al., 2000).

During aging, GDNF protein and GDNF mRNA expression have shown differential regulation patterns in central and peripheral systems. While GDNF mRNA decreases with age in the mouse striatum (Blum and Weickert, 1995), it is upregulated in skeletal muscle (Ulfhake et al., 2000), an observation which was seen in denervated skeletal muscle with disease. From this, it can be suggested that aging NMJ may experience denervation and that increased GDNF expression might be promoting reinnervation (Ulfhake et al., 2000; Edström et al., 2007). However, GDNF protein content has been found to decrease with age (Nagano and Suzuki, 2003; McCullough et al., 2011). Likewise, GFRα-1 and RET expressions are increased in aging motor neurons and in dorsal root ganglions (Bergman et al., 1999; Ulfhake et al., 2000). Neurotrophin tyrosine kinase receptors have also been found to be downregulated in aging motor neurons, implying a weakened signal transduction of neurotrophins with age-related neurodegeneration (Dragunow, 1998; Bergman et al., 1999; Connor and Johnson et al., 1999). With aging, some of the main changes that occur are loss of motor neurons (Jacob, 1998) and loss of input to cell bodies of motor neurons (Kullberg et al., 1998). Decreased levels of GDNF may be devastating for motor neuron function and structure with aging; however increased physical activity may be a mechanism to combat these changes.

Conclusions

GDNF plays an important role for the development, maintenance, and survival of the neuromuscular system. GDNF causes hyperinnervation and continuous synaptic remodeling at the NMJ (Nguyen et al., 1998; Keller-Peck et al., 2001; Zwick et al., 2001; Zhao et al., 2004), and enhances motor and sensory nerve regeneration after injury (Houenou et al., 1996; Michalski et al., 2008). Furthermore, GDNF potentiates transmitter release and synaptic transmission (Wang et al., 2002) and enhances AChR insertion on postsynaptic cells (Yang et al., 2001; Yang and Nelson, 2004). GDNF expression has been shown to be upregulated after tissue damage, with the level of GDNF mRNA increasing after nerve injury (Naveilhan et al., 1997) in denervated muscle and in neuromuscular diseases (Lie and Weis, 1998; Suzuki et al., 1998a), suggesting a role in reinnervation and synaptic maintenance. Since GDNF expression has been found to decrease with age (Nagano and Suzuki, 2003; McCullough et al., 2011), this could negatively impact motor neuron structure and function because GDNF is known as one of the most potent survival factors for motor neurons (Henderson et al., 1994; Oppenheim et al., 1995). Increasing an individual's level of exercise and/or physical therapy may be a countermeasure to increase GDNF expression and protect motor neurons from degeneration.

Abbreviations

Acetylcholine Receptor (AChR); Amyotrophic Lateral Sclerosis (ALS); Artermin (ARTN); Brain-Derived Neurotrophic Factor (BDNF); Central Nervous System (CNS); GDNF Family Receptor α (GFR-α); Glial Cell Line-Derived Neurotrophic Factor (GDNF); Glycosyl-Phosphatidylinositol (GPI); Insulin-Like Growth Factor (IGF); Leukaemia Inhibitory Factor (LIF); Nerve Growth Factor (NGF); Neuromuscular Junction (NMJ); Neurotrophin-3 (NT-3); Neurotrophin-4 (NT-4); Neurturin (NTRN); Nicotinic Acetylcholine receptor (nAChR); Peripheral Nervous Systems (PNS); Persephin (PSPN).

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