Effects of exercise on tropomyosin-related kinase B receptor isoforms and ligands in skeletal muscle

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Abbreviations

BDNF	brain-derived neurotrophic factor
dNTP	2'-deoxynucleoside 5'-triphosphate
GAPDH	glyceraldehyde-3 phosphate dehydrogenase
Ig-C2	immunoglobulin-like C2 domain
MAPK	mitogen-activated protein kinase
MG	medial gastrocnemius
mRNA	messenger ribonucleic acid
NGF	nerve growth factor
EDL	Extensor Digitorum Longus
NT-3	neurotrophin 3
NT-4/5	neurotrophin-4/5
DNA	deoxynucleic acid
R_0	theoretical starting fluorescence in a polymerase chain reaction
RER	rough endoplasmic reticulum
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
SOL	soleus
TGN	trans-golgi network
Trk	tropomyosin-related kinase
TrkB	tropomyosin-related kinase B
NAD+	nicotinamide adenine dinucleotide

MHC	myosin heavy chain
ANOVA	analysis of variance

Abstract

Exercise is a potent stimulus for the modification of gene expression in the neuromuscular system. Despite a surge of research involving the activity-dependent expression of neurotrophins in the central nervous system, little is understood of the effect of exercise on neurotrophin expression in skeletal muscle. Two members of the neurotrophin family, brain-derived neurotrophic factor (BDNF) and neurotrophin-4/5 (NT-4/5) increase in expression following periods of elevated muscular activity but have not been thoroughly investigated under forced exercise. To test if treadmill exercise could be used as a model to study expression of BDNF, NT-4/5 and their receptor tropomyosin-related kinase B (trkB), real-time reverse transcription polymerase chain reaction was performed on medial gastrocnemius and soleus muscle extracted from Sprague Dawley rats that performed ten days of treadmill exercise. Treadmill exercise did not alter expression of the genes of interest in either soleus or medial gastrocnemius. Expression of the control genes, β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were stable following exercise however β-actin was an ideal control gene as it was less variable and more stable following exercise. There was a significant effect of muscle type on expression of the control genes GAPDH ($p<0.001$) and β-actin ($p<0.001$). Comparison of neurotrophin gene expression between medial gastrocnemius and soleus was not possible as expression of GAPDH and β-actin were not equivalent between muscle types. In the present study, ten days of treadmill exercise did not elicit changes in BDNF, NT-4/5 and TrkB expression in rat hindlimb skeletal muscle. Future work is required to identify appropriate control genes for relative quantification of gene expression between muscles of differing phenotypes.

Introduction

The adaptive capability of the human body following any form of physical training is an area of intensive investigation. While the adaptations that occur following endurance exercise have been well documented in human and animal models with respect to muscle (for review see Hawley, 2002), recent evidence suggests that components of the nervous system are also influenced (Beaumont and Gardiner, 2002; Beaumont and Gardiner, 2003; Farmer et al., 2004; Vaynman et al., 2006). A family of molecules referred to as neurotrophins that consists of brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin 3 (NT-3) and neurotrophin 4 (NT-4/5), exert potent effects on the properties of the neuromuscular system (Belluardo et al., 2001; Carrasco and English, 2003; Gonzalez and Collins, 1997). It has been theorized that muscle-derived neurotrophins could be integral in the adaptive response of multiple components of the neuromuscular system to exercise (Beaumont and Gardiner, 2003; Munson et al., 1997b). The significance of neurotrophin expression in skeletal muscle is unknown and while accounts of increased BDNF expression are plentiful (Gomez-Pinilla et al., 2001; Gomez-Pinilla et al., 2002; Ying et al., 2003), consistent accounts of activity-dependent expression of NT-4/5 are sparse (Funakoshi et al., 1995; Zhang et al., 2000; Zhang et al., 1999). The majority of the literature in support of activity-dependent neurotrophin expression in skeletal muscle involves long-term voluntary exercise (Gomez-Pinilla et al., 2002; Ying et al., 2003) while the effects of exercise interventions of shorter duration are not as thoroughly understood (Gomez-Pinilla et al., 2001).

Neurotrophin synthesis, structure and signaling

Much of what is known about the structure of the neurotrophins is based on studies of the first-characterized neurotrophin NGF. Mature neurotrophin monomers display a moderate level of sequence homology (51-65%) with the regions of highest homology closely situated to six invariant cysteine residues (Ip et al., 1992; Leibrock et al., 1989; Maisonpierre et al., 1990). Examination of the tertiary structure of NGF reveal that the six cysteine residues are linked through disulfide bonds to form a knot-like structure which classifies NGF as a member of the cysteine knot super family (Holland et al., 1994). Given the conservation of the cysteine residues across neurotrophin family members, it is likely that they also exhibit the cysteine knot structure (Leibrock et al., 1989).

Neurotrophin proteins are produced as pre-pro-neurotrophin precursors (pre-pro-NT) that undergo proteolytic cleavage to yield a mature neurotrophin monomer. The pre sequence acts as a signal peptide and is essential for the formation of the neurotrophin by rough endoplasmic reticulum (RER) ribosomes (Halban and Irminger, 1994). The significance of the pro sequence has been implicated in formation of the tertiary protein structure indicated by greater refolding yields for pro-NGF than NGF alone (Rattenholl et al., 2001). Following proteolytic cleavage of the pre-domain and pro-domain assisted folding (Rattenholl et al., 2001), neurotrophins form homo- or heterodimers and are transported to the trans-golgi network (TGN) (Lessmann et al., 2003). Once in the TGN, the pro-neurotrophin can enter two different secretory pathways; constitutive secretion in which the pro sequence is cleaved by protein convertases resident in the TGN before packaging into secretory granules, or regulatory secretion in which pro-neurotrophins are

cleaved by protein convertases in secretory granules outside the TGN (Halban and Irminger, 1994; Lessmann et al., 2003). Neurotrophins secreted via the constitutive pathway are believed to act as survival factors, while regulatory secretion of the neurotrophins could allow for neuropeptide activity (Merighi, 2002). The significance of either pathway in skeletal muscle is not known. Following cleavage of the pro region in immature secretory granules or within the TGN, the mature neurotrophin protein is complete and exists as homo- or heterodimers with other neurotrophins.

Neurotrophins are capable of binding to two different transmembrane receptors, the tropomyosin-related kinase (Trk) receptor family (Klein et al., 1992; Klein et al., 1991) and the p75 neurotrophin receptor (Chao and Hempstead, 1995; Rodriguez-Tebar et al., 1992). Trk receptors are tyrosine kinases with an extracellular domain which includes three leucine-rich motifs with two immunoglobulin-like C2 type (Ig-C2) domains (Haniu et al., 1995) anchored to a cytoplasmic region with a kinase domain via a transmembrane domain. The Ig-C2 domains are essential for ligand binding and have also been implicated in prevention of spontaneous dimerization of the receptor (Arevalo et al., 2000; Urfer et al., 1995). The Ntrk2 gene encodes full length trkB and two truncated isoforms of the receptor (Middlemas et al., 1991) capable of initiating intracellular signaling (Baxter et al., 1997; Reichardt, 2006) despite an abbreviated cytoplasmic domain lacking kinase activity (Klein et al., 1990).

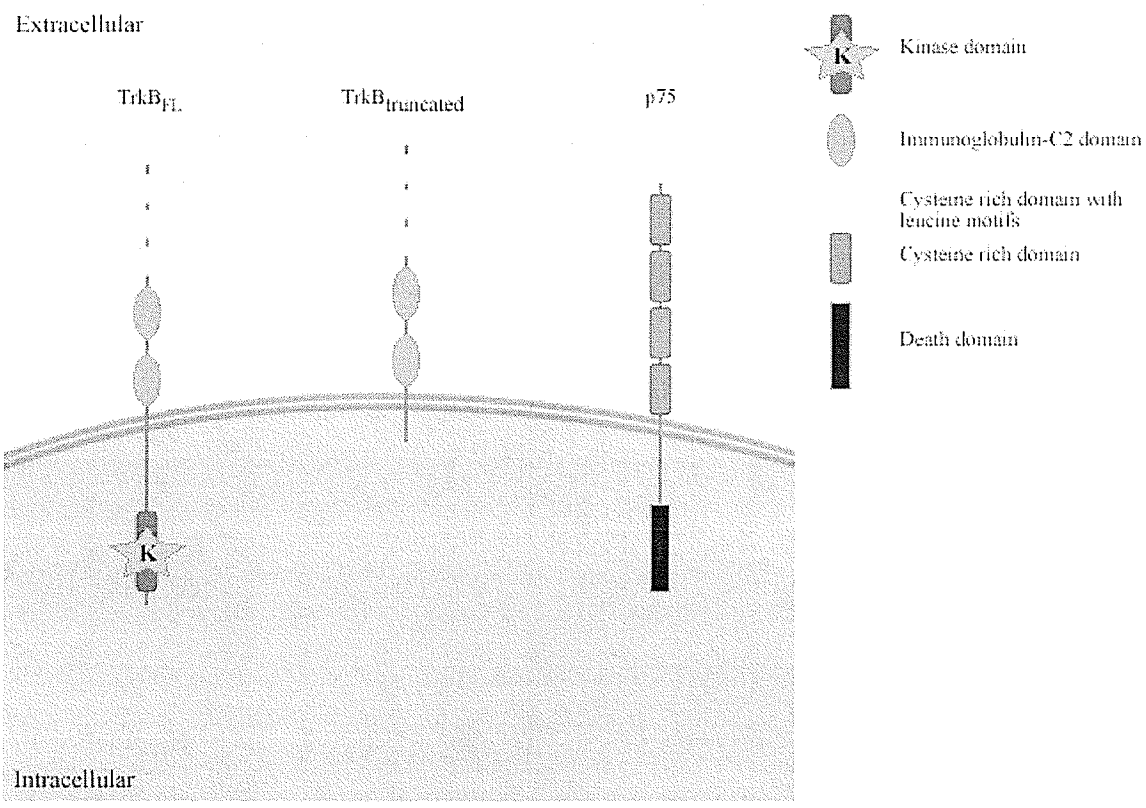


Figure 1 Schematic representation of neurotrophin receptors
Truncated trkB receptors lack the intracellular kinase domain of the full length trkB receptor. The p75 receptor bares no structural resemblance to the trk family of receptors. Adapted from Yano & Chao (2000)

The p75 receptor, a member of the tumor necrosis factor receptor family, shares no structural or sequence similarities with the trk receptors with respect to ligand binding or cytoplasmic domains (Yano and Chao, 2000) (Figure 1). The intracellular domain of the p75 receptor is highly conserved (Liepinsh et al., 1997) while the extracellular domain contains four cysteine-rich repeats characteristic of the tumor necrosis receptor family (Baldwin et al., 1992). The precise physiological role of the p75 receptor is unclear but the receptor appears to modulate trk receptor affinity (Benedetti et al., 1993) and free radical defense (Tyurina et al., 2005). The p75 receptor exhibits two contrary actions in that it is capable of inducing apoptosis or promoting cell survival (for review see Nykjaer et al., 2005). The significance of p75 in skeletal muscle is not understood but

the receptor is believed to be important for BDNF signaling in skeletal muscle satellite cells (Mousavi and Jasmin, 2006).

Our understanding of neurotrophin-activated signaling cascades is based primarily on neuronal tissue while neurotrophin signaling in muscle has not been investigated. Neurotrophin binding to trk receptors initiates ligand-induced dimerization that is capable of activating several well characterized signaling pathways. Binding to p75 is more complex and it has been theorized that neurotrophins cause dissociation of p75 multimers which allow monomers of p75 to interact with trk monomers (Reichardt, 2006). When bound to trk receptors, transphosphorylation of specific tyrosine residues recruits additional adaptor proteins and initiates downstream signaling cascades. In neuronal populations, trk receptors activate pathways involved in neuronal differentiation and survival including mitogen activated protein kinases (MAPK), the phosphatide 3-kinase/protein kinase B cascade and the phospholipase Cγ/protein kinase C cascade (Middlemas et al., 1994). The in vivo effects of p75 binding, on the other hand, remain unknown (for review see Arevalo and Wu, 2006; Barbacid, 1994). Activation of the MAPK cascade in the lumbar spinal cord following exercise is dependent on BDNF and trkB (Vaynman et al., 2003) but it is not known if similar signaling mechanisms exist in muscle.

Expression of BDNF, NT-4/5 and trkB in skeletal muscle

Determining the expression of neurotrophins has been troublesome because of conflicting reports of their presence in skeletal muscle. Often, this is due to interspecies differences (Pitts et al., 2006), however, issues of developmental timing of expression

and technical limitations of experimental methods are also confounding factors (Liem et al., 2001).

Brain-derived Neurotrophic Factor

BDNF is present at low levels in mature skeletal muscle (Funakoshi et al., 1993; Gomez-Pinilla et al., 2001; Liem et al., 2001) and has much higher expression during embryonic development than during postnatal development (Funakoshi et al., 1995; Griesbeck et al., 1995). One study, however, has reported BDNF increasing expression in soleus into postnatal life (Nagano and Suzuki, 2003).

The cellular localization of BDNF within skeletal muscle is not definitive. Initial theories implicated BDNF as a muscle-derived trophic factor for motor neurons (DiStefano et al., 1992), however reports of subsynaptic BDNF are inconsistent (Liem et al., 2001; Mousavi and Jasmin, 2006). The ability of a mature myofiber to express BDNF is debatable. Liem et al. (2001) found BDNF expression within myofibrillar nuclei in both the sub-junctional and extrajunctional compartments. The cytoplasm surrounding the sub-junctional nuclei was devoid of BDNF mRNA while the nuclear regions of extrajunctional nuclei were not, suggesting differing regulation of BDNF expression by subcellular localization. This is in agreement with Copray et al. (2000) who found cytoplasmic BDNF mRNA surrounding fibrils and in nuclear regions, but not in association with mitochondria. Other authors have failed to identify BDNF expression within mature myofibers but found expression localized to surrounding satellite cell populations (Mousavi and Jasmin, 2006). This conflicts with Liem et al. (2001) who suggest that satellite cell expression does not occur in normal muscle but only under pathological conditions. Consequently, activated satellite cells have greater expression

than quiescent cells (Copray et al., 2000). Mousavi and Jasmin (2006) found a strong positive correlation between BDNF and Pax3 mRNA demonstrating the dependence of BDNF on total satellite cell content. This is refuted by embryonic data showing no difference in BDNF expression between muscles of different contractile (fast or slow) or anatomical (flexion or extension) function (Vernon et al., 2004) which differ in satellite cell content. This relationship may not exist in mature animals as profound changes in neurotrophin expression occur in the transition to postnatal life (Funakoshi et al., 1995; Griesbeck et al., 1995).

Neurotrophin-4/5

Quantification of NT-4/5 has been described as less problematic than other neurotrophins (Pitts et al., 2006) although its expression in skeletal muscle is contentious. Messenger RNA is readily detected in skeletal muscle (Funakoshi et al., 1995; Funakoshi et al., 1993) while protein levels remain problematic (Zhang et al., 2000; Zhang et al., 1999). Nevertheless, there are accounts of NT-4/5 mRNA and protein expression in human (Walker and Schon, 1998) and rodent skeletal muscle (Funakoshi et al., 1995; Funakoshi et al., 1993; Griesbeck et al., 1995; Omura et al., 2005). Neurotrophin-4/5 is the predominate trkB ligand in skeletal muscle as expression increases into post natal life (Funakoshi et al., 1995; Griesbeck et al., 1995) . Furthermore, the association of NT-4/5 and trkB in skeletal muscle is supported by localization studies (Wiedemann et al., 2006) while BDNF is believed to act via the p75 receptor (Mousavi and Jasmin, 2006).

In contrast to BDNF, NT-4/5 is localized within myofibers and is often associated with truncated trkB receptors localized to mitochondrial membranes (Wiedemann et al., 2006). A fiber-type-dependent expression of NT-4/5 has not been resolved with reports

indicating either higher expression in type II (Sakuma et al., 2001) or type I fibers (Funakoshi et al., 1995).

Tropomyosin-related Kinase B

The presence of the full length trkB receptor and the localization of any trkB isoforms in skeletal muscle are contestable. Full length and truncated trkB isoforms have been identified in skeletal muscle and cultured myotubes of the rat (Gonzalez et al., 1999; Wells et al., 1999) and in human skeletal muscle (Shelton et al., 1995; Yamamoto et al., 1996), however recent work has failed to locate $trkB_{FL}$ using reverse-transcription polymerase chain reaction (RT-PCR) in adult rodent diaphragm, cultured myoblasts, or four-day differentiated myotubes (Mousavi and Jasmin, 2006). Truncated isoforms dominate expression in the mature rat vastus muscle (Wiedemann et al., 2006). This is in accordance with previous work where total trkB was very low and full length trkB was nonexistent, indicating truncated trkB isoforms dominate expression in muscle (Funakoshi et al., 1993).

The localization of trkB in adult skeletal muscle remains elusive. TrkB has been localized to neuromuscular junctions (Sakuma et al., 2001); specifically postsynaptic membranes around acetylcholine receptor clusters (Gonzalez et al., 1999) however truncated isoforms have recently been localized to mitochondrial membranes (Wiedemann et al., 2006). The localization of $trkB_{FL}$ with neuromuscular junctions has been theorized to be activity-dependent, decreasing with denervation and activity blockade (Pitts et al., 2006).

Activity modulates BDNF, NT-4/5 and TrkB expression

Supplementary neuromuscular activity increases expression of neurotrophins throughout the neuromuscular system. Treadmill exercise (TE) and voluntary wheel running (VWR) have been shown to regulate the expression of various neurotrophins in the central nervous system and there is a small body of literature suggesting skeletal muscle neurotrophin expression is also influenced by activity (Gomez-Pinilla et al., 2001; Skup et al., 2002; Vaynman et al., 2003).

Brain-derived Neurotrophic Factor

The expression of BDNF in the nervous system following exercise has received much attention in the literature. Voluntary wheel running over variable time periods from six hours to four weeks produces marked increases in BDNF mRNA and in the hippocampus, motor cortex and both hemispheres of the brain (Adlard and Cotman, 2004; Adlard et al., 2005; Adlard et al., 2004; Berchtold et al., 2002; Berchtold et al., 2001; Griesbach et al., 2004; Kim et al., 2005; Klintsova et al., 2004; Neeper et al., 1995; Neeper et al., 1996; Oliff et al., 1998; Russo-Neustadt et al., 2000; Tong et al., 2001; Vaynman et al., 2003; Vaynman et al., 2004a; Vaynman et al., 2004b; Widenfalk et al., 1999). The early exercise-induced increase in BDNF mRNA occurs through expression of exons I and III (Russo-Neustadt et al., 2000; Tong et al., 2001) while exons II, IV and V increase after longer exercise periods (Adlard et al., 2004). There is a positive activity-dependent expression of BDNF mRNA and protein following exercise (Adlard et al., 2004; Neeper et al., 1995; Oliff et al., 1998; Widenfalk et al., 1999). To further substantiate this relationship, animals who were exercised for five weeks had decreased BDNF mRNA after five to ten days of detraining (Widenfalk et al., 1999). Animals who

had exhibited the highest running distance during the five week exercise period experienced the greatest declines in BDNF during the cessation period. Interestingly, hippocampal expression of BDNF mRNA has a high negative correlation with escape times from the Morris Water Maze, indicating animals with high spatial learning abilities have higher levels of BDNF (Vaynman et al., 2004b).

Exercise-induced increases in BDNF mRNA and protein levels have been detected in the lumbar spinal cord. Following a structured treadmill training program, BDNF mRNA has been found to increase in large neurons of Lamina IX (Macias et al., 2002) with BDNF protein levels increased in the spinal gray matter, fibers in close opposition to large neurons, and in dendritic fibers (Skup et al., 2002). Short-term treadmill training resulted in increased BDNF immunoreactivity in fibers of the gray and white matter and a shift in intracellular localization of BDNF deposits towards distal regions of the cell (Macias et al., 2005). Elevations in BDNF mRNA and protein have been detected following three days of voluntary exercise or five days of treadmill training confined to motoneurons, axons and astrocytes of the ventral horn (Gomez-Pinilla et al., 2001; Gomez-Pinilla et al., 2002). The increase in BDNF protein induced by seven days of treadmill walking has been show to be as great as when exercise was performed for a 28 day period (Macias et al., 2007). When a region of the spinal cord is isolated via transection at T12 and bilateral dorsal rhizotomy in the lumbar region, BDNF mRNA and protein levels are decreased in the isolated lumbar spinal cord. Conversely, cervical regions have increased BDNF expression due to the increased reliance on the upper limbs for locomotion (Gomez-Pinilla et al., 2004). Following hemisection, BDNF mRNA and protein levels decline in the lumbar spinal cord whereas exercised, hemisected animals

have higher levels of BDNF than sedentary intact controls (Ying et al., 2005). Other authors have failed to find significant increases in BDNF protein following 21 days of voluntary exercise and have also noted a region-dependent regulation of BDNF exons with exons I, III and V increasing in the lumbar spinal cord but not within the thoracic cord (Perreau et al., 2005). Similar to the hippocampal BDNF expression, strong positive correlations with total running distance have been found within the spinal cord using voluntary wheel running (Gomez-Pinilla et al., 2002; Ying et al., 2005). This effect has not been demonstrated for BDNF protein levels following treadmill exercise in the lumbar spinal cord (Macias et al., 2007). Skeletal muscle BDNF mRNA and protein expression increases following three days of voluntary exercise and after five days of treadmill exercise (Gomez-Pinilla et al., 2001; Gomez-Pinilla et al., 2002).

Neurotrophin 4/5

There is little work on expression of NT-4/5 in the central nervous system following exercise. NT-4/5 immunoreactivity was greater in the white matter fibers of L3 and L4 in animals that underwent long-term treadmill training (Skup et al., 2002). Trained animals also had higher numbers of NT-4/5 positive fibers that was particularly evident in the ventral funiculi. Through double-labeled immunohistochemistry, the authors found that the enhanced NT-4/5 staining was present in both astroglial and non-astroglial fibers (Skup et al., 2002).

While some describe NT-4/5 as the primary neurotrophin in the intact adult neuromuscular system (Griesbeck et al., 1995), an activity-dependent expression of NT-4/5 has not been investigated following exercise in skeletal muscle. Muscle NT-4/5 mRNA has been shown to increase following electrical stimulation of the sciatic nerve

and to decrease following denervation or axotomy (Funakoshi et al., 1995; Sakuma et al., 2001). Blockade of neuromuscular transmission with α-bungarotoxin reduces muscle NT-4/5 mRNA (Funakoshi et al., 1995). Under stimulation and denervation conditions similar to Funakoshi et al (1995), NT-4/5 protein levels did not change in gastrocnemius and were difficult to detect despite a highly sensitive immunoassay (Zhang et al., 2000). These results are contrasted by Omura et al (2005) who successfully detected NT-4/5 protein in rat soleus (10 $pg \cdot mg^{-1}$) and found significant decreases following neurapraxia, axonotmesis and neurotmesis. Skeletal muscle biopsies from aerobically trained humans do not display increased NT-4/5 protein compared to sedentary individuals (Walker and Schon, 1998). An activity-dependent relationship of protein expression is further refuted by Deschenes et al (2003), who found no significant change in NT-4 following a period of hindlimb unloading which would be expected to produce a decline in NT-4. When increased activity is induced through surgical ablation of synergists, NT-4/5 mRNA has been shown to decrease despite significant increases in muscle activity and weight (Sakuma et al., 2001).

Tropomyosin-related kinase B

There is a positive activity-dependent expression of the primary receptor for BDNF and NT-4/5, trkB in the central nervous system. Both VWR (Gomez-Pinilla et al., 2002; Vaynman et al., 2003; Widenfalk et al., 1999) and treadmill training (Macias et al., 2005; Skup et al., 2000; Skup et al., 2002) have produced significant increases in trkB mRNA in regions of the brain and spinal cord. In exercised animals, deprivation of exercise decreases trkB mRNA in the brain while expression in the spinal cord was resistant to decreased activity (Widenfalk et al., 1999).

There is considerably less data on the effects of exercise on trkB expression in skeletal muscle. Gomez-Pinilla et al (2002) found voluntary wheel running over a three-day period to increase trkB mRNA in soleus 185%. By seven days of exercise trkB mRNA had returned to baseline levels, indicative of a time-dependent effect of exercise on trkB expression.

Implications for neurotrophins in the adaptation of skeletal muscle to exercise

Exercise is a potent stimulus for modification of the morphological, phenotypic and enzymatic characteristics of skeletal muscle. Endurance training results in increased expression of MHC_I and MHC_{IIa} with reductions in MHC_{IIb} isoforms (Allen et al., 2001; Demirel et al., 1999; Fuller et al., 2005; Kariya et al., 2004) and increased capillary supply to individual oxidative fibers (Brown et al., 1992; Ishihara et al., 1991). Enzymatic adaptations to exercise include increased amount and activity of mitochondrial enzymes, specifically cytochrome oxidase (Beaumont and Gardiner, 2003), citrate synthase (Baldwin et al., 1977; Demirel et al., 1999; Fuller et al., 2005; Powers et al., 1992), phosphofructokinase and cytochrome C (Rodnick et al., 1989). Endurance exercise also increases the major skeletal muscle glucose transporter, GLUT4 (Gulve et al., 1993). It is obvious that exercise induces multiple changes in the structural and functional parameters of skeletal muscle and there are data to suggest a role for muscle-derived neurotrophin expression in inducing such adaptations.

Recent data suggest that there is potential for an interesting link between neurotrophin expression and expression of the slow MHC isoforms. During post-natal development the soleus muscle undergoes a rapid shift from fast MHC to slow MHC expression that does not occur in the absence of NT-4/5 (Carrasco and English, 2003).

This effect is specific to NT-4/5 and dependent on trkB, although a distinction of full-length and truncated isoforms cannot be made due to the use of an immunoadhesin chimera. The authors concluded that the effects of NT-4/5 were produced through retrograde neuronal transport as $trkB_{FL}$ is localized to NMJs (Funakoshi et al., 1993). Recent data suggest that NT-4/5 is located within the myofiber and associates with truncated trkB receptors on the mitochondrial membrane (Mousavi and Jasmin, 2006). This indicates a potential autocrine action in which NT-4/5 acts on muscle through truncated trk receptors. As endurance training promotes expression of slow MHCs, it is possible that the activity-dependent expression of NT-4/5 observed by Funakoshi et al (1995) following stimulation would occur following endurance training, and the increase in NT-4/5 could be related to expression of MHC_I.

Simon et al. (2003) tested the ability of neurotrophins to promote preservation and recovery of muscle fiber size and phenotype following transection of the sciatic nerve. In this study, NT-4/5 had remarkable influence over the predominantly slow twitch soleus muscle but had little effect on the mass of extensor digitorum longus (EDL). Type I fibers were virtually eliminated from the soleus muscle group 120 days following denervation, however this loss was greatly reduced in the presence of NT-4/5. When NT-4/5 impregnated fibronectin conduits were inserted into a transected sciatic nerve, the fast muscle EDL displayed an increase in the proportion of type I fibers, consistent with the effects noted by Carrasco et al (2003). Carrasco et al (2003) suggest that NT-4/5 is capable of affecting all motoneurons regardless of type and imposes expression of a slower phenotype. This is consistent with NT-4/5 expression being confined to slow twitch fibers (Funakoshi et al., 1995).

There is a growing body of literature that suggests a potential role for neurotrophins in exercise-induced plasticity of the neuromuscular system. It has not been determined whether these interactions occur under normal physiological conditions. Nonetheless, these interactions currently act as a theoretical justification for the involvement of neurotrophins in the adaptive response of the neuromuscular system to exercise.

Two potential roles for muscle-derived neurotrophins

Research into the significance of muscle-derived neurotrophins has been hampered by the lack of a physiologically relevant model to study their action. Despite this, muscle-derived neurotrophins have been hypothesized to act as retrograde trophic factors for α-motoneurons (Carrasco and English, 2003; Gordon et al., 1997; Munson et al., 1997a) and as control factors for myogenic differentiation of muscle-derived satellite cells (Mousavi and Jasmin, 2006).

The retrograde trophic factor hypothesis arose from the ability of motor neurons and muscle to match their functional properties. Following an increase in activity of the motor unit, skeletal muscle increases production of certain trophic factors. These proteins diffuse across the neuromuscular junction where they are taken up and retrogradely transported to the soma of the α-motoneuron. Once in the soma, the trophic factors alter motoneuron gene expression and produce proteins that either act on the motor neuron or are orthogradely transported to act on skeletal muscle. Chronic stimulation experiments have led to the formation of this theory however these experiments cannot implicate neurotrophins specifically (Gordon et al., 1997; Munson et al., 1997b). Low frequency chronic stimulation alters the contractile and histochemical properties of medial

gastrocnemius muscle fibers (Gordon et al., 1997). Control medial gastrocnemius expressed three fiber types (type I slow oxidative, type IIa fast oxidative glycolytic, type IIb fast glycolytic) but when stimulated for 56 days expressed predominately type I and when stimulated for 76 days were completely type I slow oxidative (Gordon et al., 1997). Muscle contractile properties were shifted to resemble a more "slow-like" fiber type indicated by decreased normalized force, increased time to peak tension and an improved endurance index. Motoneuron properties were also influenced by chronic stimulation although the conversion was not as complete as seen in muscle. The implication of such work is that chronic stimulation results in a coordinate adaptation of all components of the motor unit with adaptation in muscle preceding that of the motoneuron. Gordon et al (1997) conclude that a retrograde loop involving neurotrophins could be responsible for the coordinate adaptation of muscle and motoneuron. The effects of chronic stimulation on muscle-derived neurotrophin expression or retrograde transport have not been evaluated, however brief periods of stimulation have profound effects neurotrophin expression in skeletal muscle (Funakoshi et al., 1995).

Support for the retrograde trophic factor model can be found in studies that supply exogenous neurotrophins to skeletal muscle. Following insertion of a BDNF saturated gel foam into medial gastrocnemius, the biophysical properties of the innervating motoneurons were significantly different (Gonzalez and Collins, 1997). Medial gastrocnemius motoneurons were found to be more excitable and inferred to be smaller in size based on total cell capacitance. Unfortunately, uptake and transport of BDNF was not confirmed making it difficult to definitively attribute alterations in motoneuron properties to retrograde transport of BDNF. It is possible that the presence of exogenous

BDNF could have altered the production of another muscle-derived factor that acted to change motoneuron properties. Neurotrophin-4/5 was not investigated in this study and as the effects were believed to be mediated through the trkB neurotrophin receptor it is possible that NT-4/5 could have been involved in the effects noted. Whether a similar result could be obtained under physiological circumstances is unknown as exogenous application does not conform to normal regionalization of expression. Recent work has failed to locate BDNF in synaptic regions making the retrograde transport of BDNF in a mature intact neuromuscular junction unlikely (Mousavi and Jasmin, 2006). Much work is needed to substantiate the idea of the retrograde-trophic factor hypothesis in the mature neuromuscular system.

An alternate hypothetical role for muscle-derived neurotrophins, specifically BDNF, is the regulation of satellite cell activity during regeneration and repair of skeletal muscle. While the retrograde trophic factor hypothesis favors a sub-synaptic localization of BDNF, Mousavi and Jasmin (2006) did not detect BDNF within muscle fibers or synaptic regions but instead localized BDNF to satellite cells adjacent to the myofibers. In cell culture experiments BNDF acted to inhibit myogenic differentiation of satellite cells and appeared to be an integral factor in maintaining progenitor cell population (Mousavi and Jasmin, 2006). In this context it would appear that increased BDNF signals could interfere with the regeneration and repair of muscle fibers damaged by exercise. Other authors have hypothesized that induction of BDNF expression following muscle damage could serve to maintain the integrity of the neuromuscular connections (Copray et al., 2000). While studying the effects of electrical stimulation in slow twitch muscle, Copray and colleagues (2000) discovered increased muscle damage and elevated BDNF

mRNA in damaged fibers of diabetic rats. The increased BDNF signal was localized within myofibers and also in activated satellite cells which complicates the current understanding of BDNF localization and its role in muscle repair and regeneration. In contrast Funakoshi and colleagues (1995) found that electrical stimulation resulted in a reduction in BDNF in medial gastrocnemius muscle fibers. As the BDNF signal is proportional to satellite cell content and decreases with myogenic differentiation (Mousavi and Jasmin, 2006), it is possible that the alterations in BDNF expression with exercise and stimulation reflect changes in the activity or number of satellite cells in skeletal muscle. Satellite cell dynamics are complicated especially when considered in the context of muscle repair following exercise or damaging contractions induced by electrical stimulation. No definitive conclusions can be drawn on how changes in BDNF expression relate to satellite cell activity in exercised or stimulated/damaged muscle.

Research into the function of muscle-derived neurotrophins is in its infancy and there is much required work left to be done. Conclusions as to the significance of this specific family of proteins is hampered by the experimental models used to study them which often rely on embryonic samples that shed little light on adult function, and the exogenous application of neurotrophins in excess of normal physiological expression. A suitable in vivo model to study neurotrophin function in skeletal muscle is required to elucidate the function of these proteins.

Real-time polymerase chain reaction to determine mRNA expression

Real-time reverse transcription polymerase chain reaction (RT-PCR) is a technique used to determine gene expression that capitalizes on the known, orderly sequence of events in the production of a protein within the eukaryotic cell (Figure 2).

Discrete sequences of DNA (genes) are copied into single stranded mRNA molecules by an RNA polymerase (transcription). The polymerase is attracted to the start site of the gene through a complex interaction of general transcription factors typically needed for transcription of all genes and a balance of many possible transcription enhancer or repressor proteins that could be present on the DNA. Within the nuclear compartment, post-transcriptional modifications of mRNA occur through the activity of the poly-A polymerase, the addition of a 5'-methyl cap and the removal (splicing) of intron sequences. These post-transcriptional modifications serve to promote nuclear export of the mature mRNA molecule. In the cytoplasm, the mRNA is processed by either free or endoplasmic reticulum-bound ribosomes which translate the nucleotide sequence into a protein polypeptide assisted by amino-acyl transfer RNAs. The polymerase chain reaction is used to assess gene expression by determining mRNA levels. This provides insight into the early events in cells that could lead to an increase in production of a specific protein.

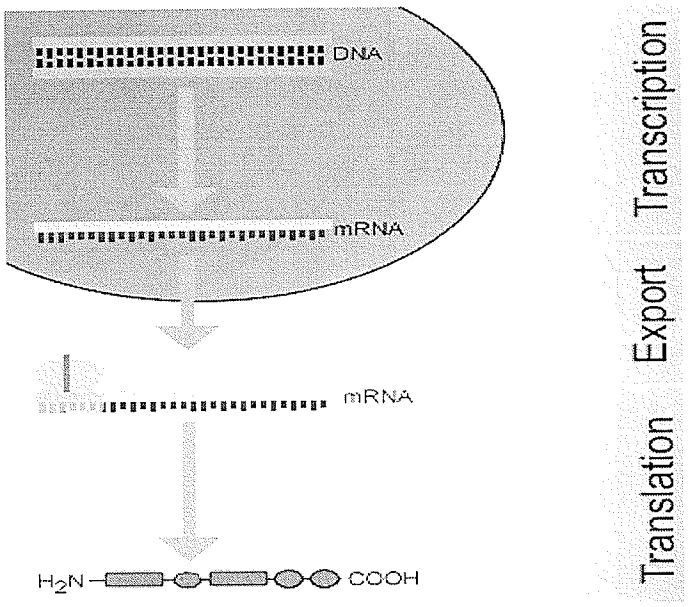


Figure 2 Production of a protein from a gene sequence located on DNA
Discrete sequences on DNA (genes) are transcribed by an RNA polymerase into single stranded mRNAs. Following export into the cytoplasm, mRNA is processed by free or bound ribosomes into polypeptide chains of amino acids (protein).

Successful analysis of mRNA from experimental samples requires much treatment before the RT-PCR reaction can be performed. A diagrammatic outline of an RT-PCR experiment is shown in Figure 3. The enzymes of the reverse transcription and RT-PCR reactions require that samples be properly lysed to ensure maximal isolation of RNA from the sample. Reverse transcription and RT-PCR reactions also require that all cellular debris (proteins, genomic DNA, and fats) be removed from the sample. Proper cell lysis and homogenization is particularly important because it ensures random fiber type sampling from whole muscle samples and eliminates positional bias and/or variability in biopsy sampling. Unfortunately eukaryotic cells lack an enzyme capable of amplification of mRNA and a synthetic enzyme has not been developed. As such, RT-PCR is dependent on the use of specific reverse transcription enzymes that are capable of producing DNA copies (cDNA) of the mRNA present in an appropriately prepared sample. Following reverse transcription, the cDNA generated can be amplified through

the use of primers (short nucleotide sequences) specific to sequences located on the cDNA and a DNA polymerase to generate template strands for amplification (the RT-PCR reaction).

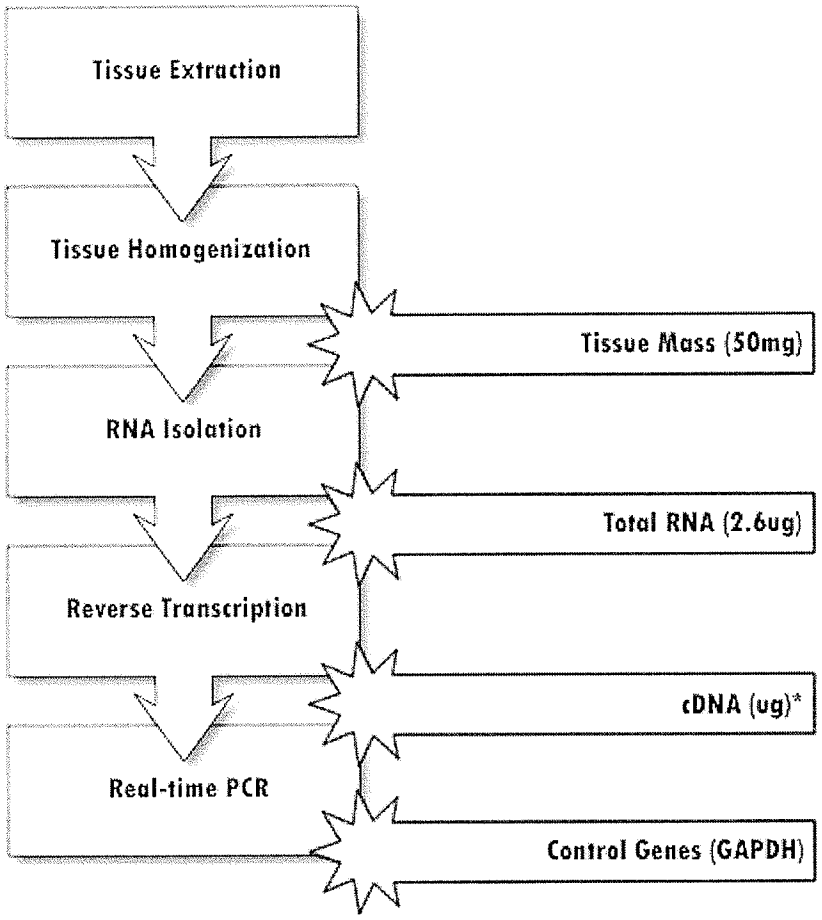


Figure 3 Stages in a real-time PCR experiment
White boxes indicate the fundamental stages in preparation of the real-time PCR reaction; grey callouts denote stages where normalization occurs and the measures taken in the present work. Real-time PCR experiments begin with extraction of tissue in RNase free conditions and quick freezing to -70°C. In fibrous tissue such as skeletal muscle manual homogenization with a mortar and pestle chilled with liquid nitrogen facilitates RNA isolation. Spectroscopy is used to determine total RNA concentration to normalize input into the reverse transcription reaction. The amount of cDNA input into PCR can be controlled and the final output of the RT-PCR reaction is normalized to a highly expressed, ubiquitous control gene. *Normalization of to the amount of cDNA was not performed in this study.

In its simplest form, the RT-PCR reaction is a combination of cDNA generated from mRNA samples, forward and reverse gene specific primers which bind to complementary regions resident on the template cDNA, a double stranded DNA binding dye (ie: SYBR green) and a DNA polymerase which performs the actual amplification of

the template cDNA. Because of the low abundance of mRNA for specific genes present in biological samples, the RT-PCR reaction amplifies the existing sample (once converted to cDNA) using a cyclical progression through three temperature stages. The reaction is heated once to 95°C for ten minutes to activate the polymerase enzyme (hot start) which is responsible for generating copies of the template cDNA. This is followed by forty cycles of two stages: 1) 95°C for 15 seconds to denature the secondary structure of the cDNA and allow the new cDNA strands generated by the polymerase enzyme to dissociate off the template strand and 2) 62°C for 1 minute to allow for primer annealing (binding to cDNA at complementary sequences) and strand extension by the polymerase enzyme. TaqMan RT-PCR chemistry adds another layer of specificity not present in the more cost effective double-stranded DNA binding dyes such as SYBR green. While both SYBR green and TaqMan detection utilize forward and reverse primers specific to certain cDNA sequences, TaqMan chemistry introduces another short (less than 30 bases) probe sequence specific to the transcript of interest. This hydrolysis probe contains a fluorochrome on the 5' end of the probe and a non-fluorescent "dark" quencher on the 3' end. During the elongation phase of the PCR reaction the 5' hydrolysis action of the Taq DNA polymerase cleaves the probe from the cDNA sequence and separates the fluorochrome from the quencher molecule, increasing the amount of fluorescence emitted by the reaction. The cycle where the fluorescence of the reaction passes a fixed fluorescence threshold is referred to as the cycle threshold and is used in data analysis to determine the level of expression of the gene of interest. A gene expressed at a high level will take fewer cycles and will therefore have a lower cycle threshold value (Ct) than a

gene expressed at a lower level which would take more cycles to pass the fluorescence threshold.

Rationale for Project

The functional significance of muscle-derived neurotrophins, particularly the trkB ligands BDNF and NT-4/5 is unknown and the effect of treadmill exercise has not been thoroughly investigated. While the expression of most neurotrophins appears to be activity-dependent in the brain and spinal cord, there is not strong evidence for NT-4/5 in these tissues. The majority of studies evaluating the effects of exercise on muscle-derived neurotrophin expression have utilized longer term voluntary exercise interventions whereas the use of forced treadmill exercise has not been as common. The effects of an intervention that taxes the metabolic and regenerative systems of skeletal muscle are of great interest given the localization of NT-4/5 with truncated trkB receptors on mitochondrial membranes (Wiedemann et al., 2006) and the expression of BDNF by satellite cells (Mousavi and Jasmin, 2006). The proposed study is intended to clarify the effect of treadmill exercise for ten days on BDNF, NT-4/5 and trkB in skeletal muscle and to determine whether treadmill exercise can be used as a model in future studies to determine the role of the trkB ligands in skeletal muscle.

Hypotheses

Effects of muscle-type on NT-4/5, BDNF and trkB

The expression of NT-4/5 and BDNF will be higher in soleus than in medial gastrocnemius. Total trkB content will be greater in soleus than medial gastrocnemius as this muscle is normally more active during locomotion (Gardiner et al., 1982).

Effects of treadmill exercise on NT-4/5, BDNF and trkB

Treadmill exercise will increase expression of BDNF, NT-4/5 and trkB as these mRNAs have shown activity-dependent expression in other tissues. The training effect will be more substantial in the soleus muscle group as this muscle is highly active during this exercise.

Methods

Exercise Animals

Female Sprague-Dawley rats 13 to 15 weeks in age were divided into two groups with one performing treadmill exercise (TE, n=12) for twelve days and another group acting as sedentary controls (CON, n=12). Animals were acclimatized to treadmill exercise for two days running at speeds of 10 to 20 $m \cdot min^{-1}$ for 20-minute periods. On the third day of exercise, animals worked up to a speed of 20 $m \cdot min^{-1}$ over 10 minutes followed by 20 $m \cdot min^{-1}$ for 15 minutes and a five-minute cool down at 10 $m \cdot min^{-1}$. On the fourth day, animals worked up to 20 $m \cdot min^{-1}$ from 10 $m \cdot min^{-1}$ over five minutes and exercised at 20 $m \cdot min^{-1}$ for 25 minutes before cooling down similar to day three. The speed and duration on the fourth day of exercise was maintained over the remaining training period. The treadmill grade was increased to 5% on day six through to the end of 12 day exercise protocol. All procedures were approved by the animal care Protocol Management and Review Committee of the University of Manitoba.

Tissue Processing and RNA Extraction

Animals were anesthetized with isoflurane before being euthanized by decapitation immediately following the final exercise session. The medial gastrocnemius

and soleus muscles were surgically extracted, immediately fresh frozen to -70°C in isopentane, and stored at -80°C until further processing.

Total RNA was extracted using a modified acid guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi, 1987) with Trizol reagent (Invitrogen) following manual homogenization of muscles in liquid nitrogen with a mortar and pestle. To ensure complete homogenization of the muscles, an electric homogenizer (Kontes) was also used with approximately 50 mg of tissue in one milliliter of Trizol reagent. RNA extraction was performed as per the manufacturer's instructions and resuspended in 20ul 0.01% DEPC ddH_2O. The concentration and purity of total RNA was determined by the absorbance at 260 nm and the ratio of the absorbance at 260nm to the absorbance at 280nm using a Nanodrop spectrophotomer (ND-1000, Nanodrop). Total RNA concentrations were utilized to normalize the input into the reverse transcription reaction to 2.6 ug of Total RNA. All samples were treated with amplification grade DNase I (Invitrogen) to prevent any residual genomic DNA from being amplified during PCR.

RT-PCR

SuperScript™ II Reverse Transcriptase kit (Invitrogen) with Oligo$(dT)_{12-18}$ primers (Invitrogen) was used according to the manufacturer's recommendations for reverse transcription. Reactions were scaled up linearly to account for the increased reaction volume due to the DNase treatment. Briefly 2.6ul Oligo$(dT)_{12-18}$ primers were combined with 2.6ug of total RNA and 2.6ul of dNTP mix (Invitrogen) and brought to a reaction volume of 24 ul with 0.1% DEPC-ddH_2O. The reaction was heated to 65°C and quick-chilled on ice before the addition of 5X first-strand buffer, 0.1M DTT (Invitrogen) and 2.6 ul RNaseOUT (Invitrogen). After a two-minute incubation at 42°C, 2.6 ul of

SuperScript™ II reverse transcriptase was added to the reaction and the reaction was incubated at 42°C for 50 minutes and finally inactivated by heating to 70°C for 15 minutes.

Real-time PCR was performed on an Applied Biosystems 7500HT Fast thermocycler. Sample cDNA (up to 9 ul) was combined with TaqMan® pre-mixed gene expression assays (1 ul) (Applied Biosystems) and 10 ul of fast master mix (Applied Biosystems) and brought to a reaction volume of 20ul with 0.01% DEPC-ddH_2O. Characteristics of the TaqMan® gene expression assays are depicted in Table 1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin were used as reference genes (Applied Biosystems) for the normalization of experimental data. Reactions were prepared in quadruplicate and run in triplicate and a maximum between-replicate difference of 0.5 CT was acceptable for statistical analysis (Scott Adams, 2006). Fast thermocycling conditions were used with extended annealing/extension phase of 35 seconds at a higher temperature of 62°C for forty cycles.

Probe (Accession #)	Location	Spans Introns	Amplicon Size
BDNF (NM_012513.2)	3561	No	142
TrkB (NM_01273.1)	1522	Yes	73
NT-4/5 (NM_013184.3)	652	No	80

Table 1 Characteristics of the TaqMan® gene expression assays
Characteristics of the preformulated TaqMan assays indicate only two of the three span intron sequences which necessitate DNase treatment of all experimental samples to prevent amplification of genomic DNA.

Data Analysis

Raw fluorescence data was exported from the thermocycler and imported into Statistica (StatSoft) for further analysis. Cycle threshold and per reaction efficiency was

determined using the online Real-Time PCR Miner application which uses a four parameter logistic model to fit experimental data (Zhao and Fernald, 2005). The initial amount of template for a particular gene (R_0) was determined and expressed as a ratio relative to β-actin expression with the formula:

$$R_0 = \frac{\frac{1}{(1+E_{target})^{CT_{target}}}}{\frac{1}{(1+E_{reference})^{CT_{reference}}}}$$

where E_{target} is the average efficiency of the target gene, CT_{target} is the cycle threshold of the target gene for that sample, $E_{reference}$ is the average efficiency of the reference gene (GAPDH) and $CT_{reference}$ is the cycle threshold for the reference gene for that sample (Zhao and Fernald, 2005). The normalized R_0 value represents the ratio of the target gene to the reference gene at the initial stages of the reaction.

Statistical Analysis

Non-normalized R_0 values were be used to determine that stability of expression of control genes GAPDH and β-actin with exercise and between muscle types while gene of interest data was normalized to β-actin. Main effects of exercise and muscle type were determined using a two-way ANOVA (muscle type x exercise condition). Tukey post hoc analysis was utilized when indicated to test for differences between means. The significance level was set to $p<0.05$ for all analyses.

Results

Control gene expression

There was no effect on expression of both β-actin (Figure 5) and GAPDH (Figure 6) with treadmill exercise. There was a main effect of muscle-type for β-actin

(F=18.8442, p<0.001) and GAPDH (F=40.3092, p<0.001) (Figure 4). B-actin expression was 184% higher in CON SOL than CON MG (p<0.01) and 161% higher than TE MG in TE SOL (p<0.05). GAPDH expression was 248% higher in CON MG than CON SOL (p<0.001) and 375% higher in TE MG over TE SOL (p<0.001). The coeffecients of variation were lower for β-actin than for GAPDH across all conditions (Table 2). All experimental data was normalized to expression of β-actin as its expression was more stable with exercise and less variable than GAPDH. Coefficients of variation taken across multiple PCR plates indicate that the RT-PCR reactions were highly reproducible (0.92% BDNF, 0.16% TrkB, 1.1% NT-4/5, 3.4% GAPDH).

	TE-Sol	**TC-Sol**	**TE-Mg**	**TC-Mg**
B-actin	48%	40%	32%	27%
GAPDH	53%	45%	52%	44%

Table 2 Coefficients of variation within experimental groups for β-actin and GAPDH R_0 values
High coefficients of variation indicate high response variability within experimental groups which strengthen any statistically significant findings (Mahoney et al., 2004).

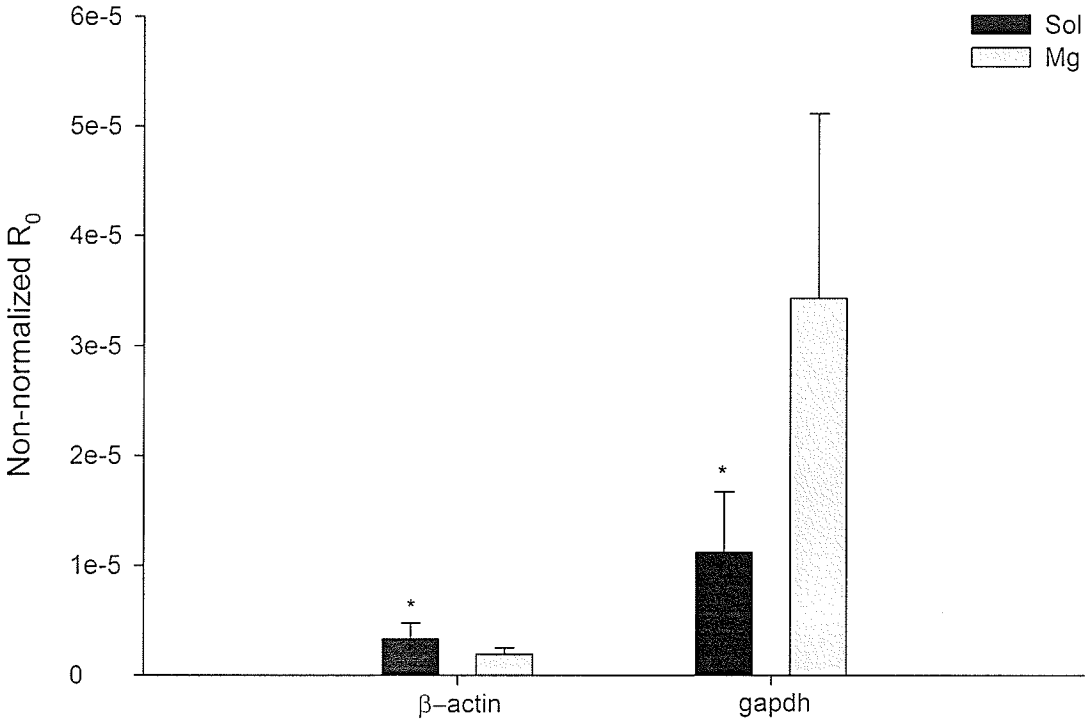


Figure 4 Effect of muscle-type on control gene expression
Non-normalized R_0 values indicate significant effects of muscle type on expression of β-actin and GAPDH. Expression of GAPDH was greater in MG than SOL while β-actin was higher in SOL. Bar graph represents mean±SD. (* denotes $p<0.001$)

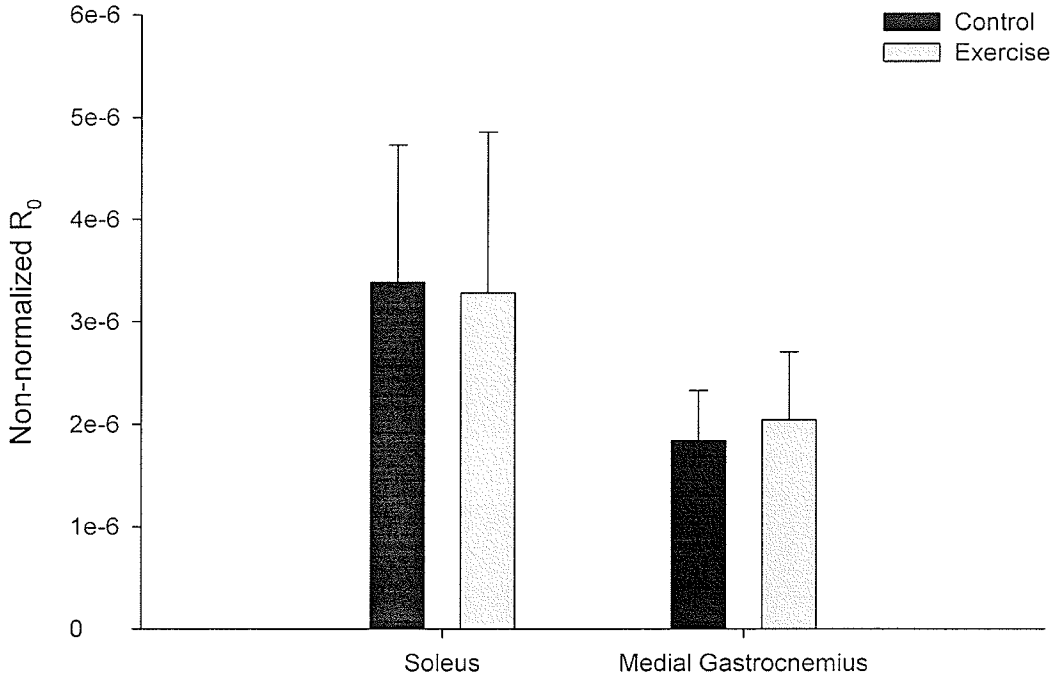


Figure 5 Non-normalized R_0 values show no effect of exercise on β-actin expression
Bar graphs represent mean±SD.

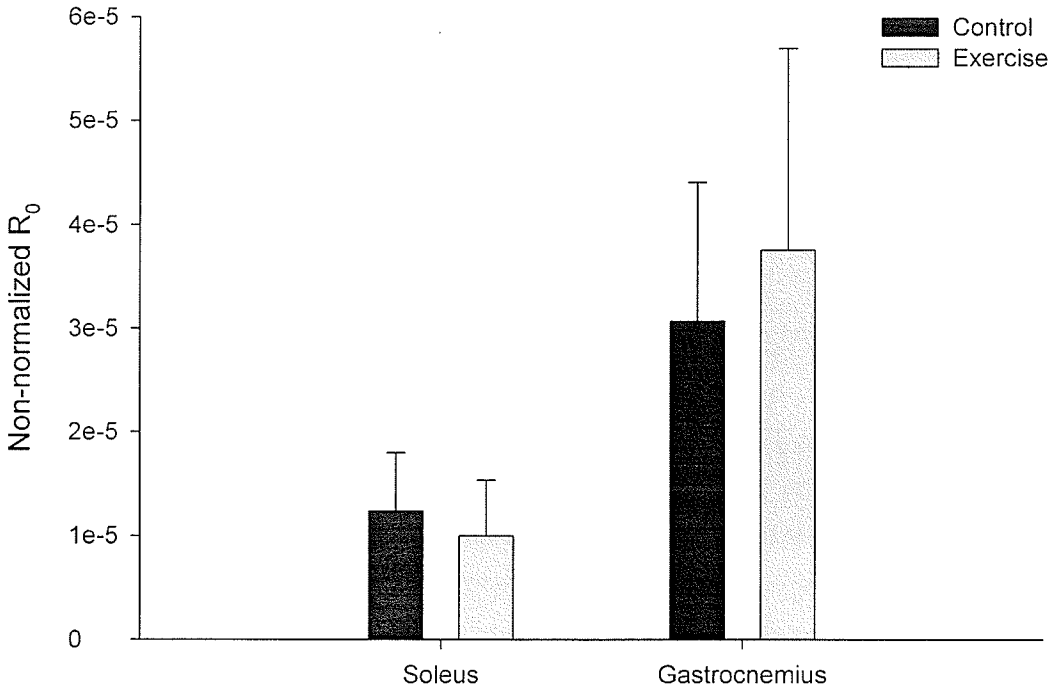


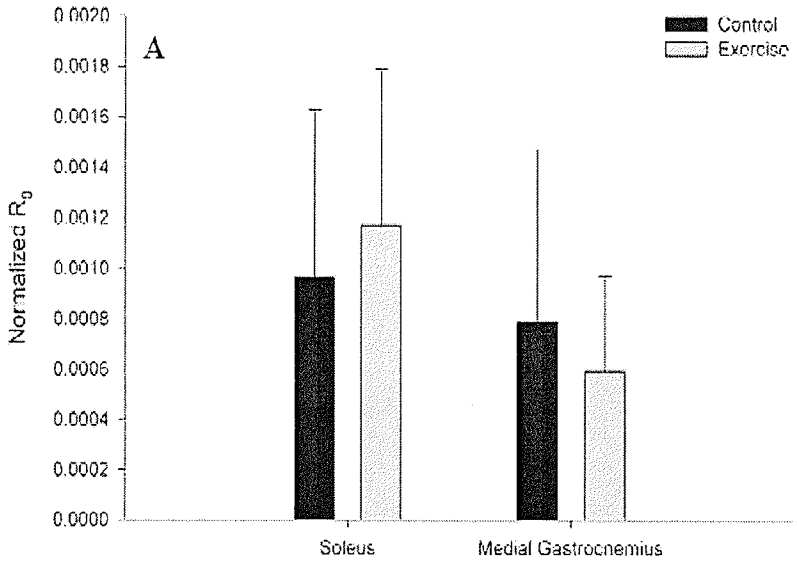
Figure 6 Non-normalized R_0 values indicate no effect of exercise on GAPDH expresion
Bar graphs represent mean±SD.

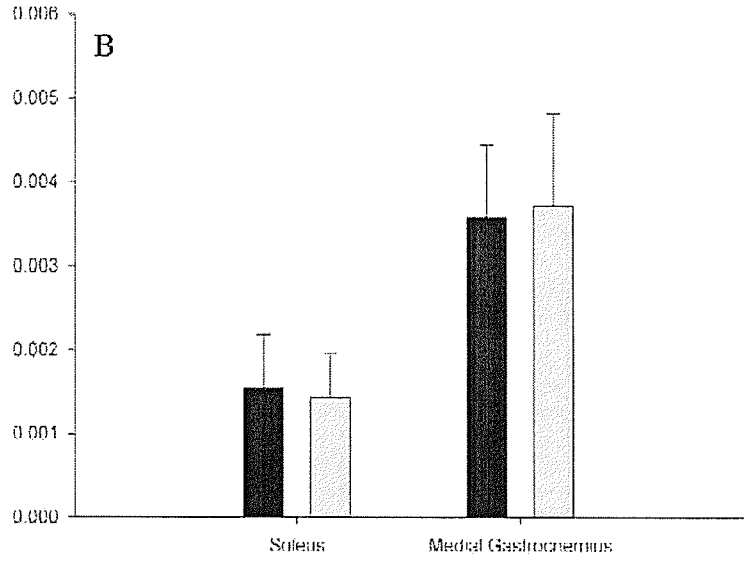
Effects of muscle-type

The difference in expression levels of GAPDH and β-actin between muscle types (Figure 4) prevented normalization of experimental data for the genes of interest. Stable, equivalent expression of control genes is required for accurate comparison of expression differences therefore inter-muscle differences could not be tested.

Effects of exercise

There was no overall effect of exercise on expression of BDNF, NT-4/5 and TrkB when normalized to β-actin (Figure 7). Due to the inequality in control gene expression between MG and SOL independent t-tests were used to test for significant difference in gene expression with exercise.





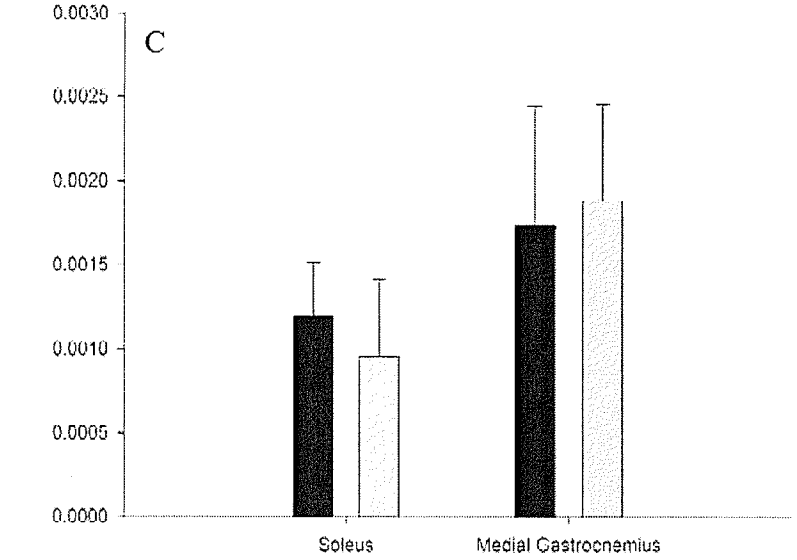


Figure 7 Exercise had no effect on expression of BDNF, NT-4/5 and trkB
A) BDNF expression was highly variable and was not significantly changed following exercise in SOL or MG. B) NT-4/5 mRNA was not significantly altered in either muscle type. C) TrkB expression was not effected by exercise in SOL and MG. Bar graph represents mean±SD.

Discussion

The use of control genes for mRNA normalization

Endogenous control genes, commonly referred to as "housekeeping" or "reference" genes, often encode proteins vital to cell maintenance and are considered to be constitutively expressed. In an RT-PCR experiment the control gene is used to account for variations in the initial RNA concentration introduced by the non-specific nature of spectroscopy. Normalization to total RNA concentration alone is an insufficient experimental control as it is often unknown if the experimental treatment has altered the proportion of mRNA present in the total RNA preparation. Commonly used control genes such as β-actin and GAPDH have been carried over from earlier techniques such as the northern blot which lack the sensitivity of the RT-PCR assay and the stability of expression is often assumed and not validated for each experiment. Stable expression of the common control genes has recently come into question as it has become apparent that various experimental treatments alter gene expression (Jemiolo and Trappe, 2004; Lowe et al., 2000; Mahoney et al., 2004). An alteration in control gene expression can produce artifactual differences in expression for genes of interest and can lead to fallacious conclusions (Mahoney et al., 2004). Stable expression of the control gene is a critical criterion in a successful RT-PCR experiment (Huggett et al., 2005).

Selection of control genes for exercise interventions

Given that exercise is a potent stimulus for the induction of expression of various genes in skeletal muscle, its effect on the expression of genes typically used to control for loading variation in RT-PCR experiments is of obvious concern. In an investigation on the stability of expression of commonly employed control genes (β-actin, GAPDH, 28S

ribosomal RNA, cyclophilin and β2-microglobulin), Mahoney et al (2004) found important differences following exercise. Following acute cycle endurance exercise, β2-microglobulin and GAPDH were not altered at 3 and 48 hours post exercise, while cyclophilin, β-actin and 28S ribosomal RNA were all increased by 48 hours post exercise. Analysis of single muscle fiber gene expression following running exercise found β-actin, β2-microglobulin and 18S ribosomal RNA to increase 4 hours post exercise in both MHC I and MHC IIa dominant fiber types while GAPDH was relatively stable in both fiber types post exercise (Jemiolo and Trappe, 2004). GAPDH stability has also been confirmed following three hours of low intensity cycle exercise in humans when normalized to single stranded DNA or RNA complementary DNA hybrids (Lundby et al., 2005). Under the same experimental conditions β-actin underwent a 2.5 fold increase in expression regardless of normalization method.

The results of the present work agree with that of Mahoney et al (2004), Jemiolo and Trappe (2004) and Lundby et al (2005) indicating GAPDH as a suitable control gene in endurance exercise interventions. This study extends previous findings which focused on acute single exercise bouts whereas the current work employed a ten-day exercise intervention. Contrary to previous investigations (Jemiolo and Trappe, 2004; Lundby et al., 2005) the current study found β-actin to be a more appropriate control gene than GAPDH based on a smaller shift with exercise both in MG and SOL but also due to lower variability of expression. This novel result could be due to the difference in length of the exercise intervention (single bout versus ten days), the choice of exercise modality (cycle versus treadmill) or the subject type (human versus animal). The disparate results

regarding the effects of exercise on expression of β-actin highlights the importance of control gene validation with each experiment.

Inter-muscle differences in control gene expression

Tissue-dependent differences in control gene expression have been noted in various studies, however specific variation between muscles has not received significant attention. Differences in control gene expression with exercise have been discovered between muscle fibers expressing MHC I and MHC IIa (Jemiolo and Trappe, 2004). Following brief treadmill exercise at 70% VO_2 max in humans, expression of GAPDH was identified as stable in MHC I and IIa fiber types when normalized to expression of β-actin. By four hours post-exercise expression of two other commonly employed control genes β2-microglobulin and 18s ribosomal RNA had increased in expression in MHC I. However in MHC II positive fibers, both GAPDH and β2-microglobulin were constant following exercise when normalized to β-actin expression. While direct comparison of gene expression between MHC I and IIa fibers were not made, it is clear that there is differential control of expression of common housekeeping genes between fibers of different phenotypes.

The present work indicates that while GAPDH and β-actin are suitable control genes for exercise intervention, the large differences in expression between medial gastrocnemius and soleus suggest they are not suitable for inter-muscle comparisons. Glyceraldehyde-3-phosphate dehydrogenase is a critical enzyme in the glycolytic pathway which catalyzes the oxidation and phosphorylation of glyceraldehyde-3-phosphate by nicotinamide adenine dinucleotide (NAD+) and inorganic phosphate. As the glycolytic capacity of muscle varies greatly with its fiber-type composition it is

reasonable to assume that there could be significant differences in expression of GAPDH between muscles of differing phenotypes. The soleus muscle has a fiber composition that is predominantly type I slow oxidative with a small population of type IIa fast oxidative glycolytic fibers while medial gastrocnemius has a more varied composition with a larger population of fast oxidative glycolytic and fast glycolytic fibers (Armstrong and Phelps, 1984). As such it is not surprising that the present work indicates that GAPDH mRNA is elevated in MG when compared to SOL. This is in agreement with Lowe et al. (2000) who found elevated GAPDH mRNA and protein in fast twitch hind-limb muscles compared to soleus. In a comparison of type I and IIa fibers using single fiber RT-PCR no significant difference in GAPDH expression was detected (Yang et al., 2006). While there was a slight insignificant elevation in expression between type IIa and type I fibers (1.35 fold, insignificant), it is likely inclusion of the fast glycolytic fibers in muscle homogenate experiments that produces the dramatic differences in GAPDH expression between muscle-types.

Actin family proteins are integral to cell motility and are ubiquitously expressed across eukaryotic cells. Of the six predominate actin isoforms four are highly expressed in smooth, skeletal or cardiac muscle while two cytoplasmic isoforms (β and γ) are expressed across all non-muscle eukaryotic cells. β-actin has been identified as a potential control gene in past studies using acute exercise (Mahoney et al., 2004) and creatine supplementation (Murphy et al., 2003), however the present work found variation in expression between muscles of different phenotypes. While related non-muscle cytoplasmic γ-actin has been localized within myofibers (Nakata et al., 2001) the precise localization of β-actin is not clear. If expression of β-actin is confined to non-

muscle cells in skeletal muscle, it is likely that the difference in β-actin expression between muscles is related to differences in the activity or number of these cells within differing muscle types. A definitive explanation for the difference in β-actin is not plausible however it is possible that the difference is related to the activity or total amount of satellite cells present in the muscle. Repair, regeneration and creation of new myofibers are contingent on the differentiation of satellite cells into myoblasts followed by fusion to form new fibers or fusion with damaged existing fibers. During this transformation cells switch from expression of non-muscle actin isoforms to expression of skeletal muscle actin isoforms. As satellite cell content varies with muscle type (Mousavi and Jasmin, 2006), it is possible that the variation in β-actin expression between muscle types could be related to satellite cell dynamics in muscle. Since cytoplasmic actins are expressed in all non-muscle cells it is possible that this variation is introduced by other cell types such as endothelial cells, epithelial cells and fibroblasts

Variability in control gene expression

An ideal control gene would be one that does not change following the experimental treatment and has low variability. In search of suitable control genes for comparisons of gene expression with aging, Touchberry et al (2006) found GAPDH to be more variable than β2-microglobulin and RNA polymerase 2a. Unfortunately specific coefficients of variation were not given for each gene and data was not presented in a linearized form preventing comparison with other work. Control gene expression following acute creatine supplementation suggests classical control genes have coeffecients of variation ranging from 20% to 39% (Murphy et al., 2003). When triplicate readings were performed across separate PCR runs overall coefficient of variation was

increased to an average of 34% from 21% when performed in the same run. The large number of samples in the present work required the use of control gene reactions to be spread across multiple PCR runs which could have inflated the inter-sample differences in gene expression and elevated the coeffecients of variations of the control genes.

There is high variability in gene expression following exercise (Jemiolo and Trappe, 2004; Mahoney et al., 2004; Psilander et al., 2003). This effect has been detected for specific myogenic regulatory factor and insulin-like growth factor I mRNAs (Psilander et al., 2003) and for many of the classical control genes (Jemiolo and Trappe, 2004; Mahoney et al., 2004). The large degree of variability is proposed to be gene specific with coefficients of variation as high as 55% for 28S ribosomal RNA (Mahoney et al., 2004). Contrary to these findings Wittwer et al (2004) found the inter-individual differences to be reduced in a group of professional cyclists compared with sedentary controls using DNA microarrays. The length of training of the professional cyclists (30000 km of cycling per year for the previous five years) was much greater than the single bout of cycling or running exercise used in Jemiolo and Trappe (2004) and Mahoney et al (2004). In the present study, exercise inflated the coefficient of variation of R_0 values by 8% for TE and CON SOL and TE MG and 5% for CON MG. This agrees with the work of Mahoney et al (2004) who noted very high variation for 28S ribosomal RNA following exercise and extends that finding by showing this high variation persists over longer training periods and for different genes. It is possible that the duration of the current exercise intervention was insufficient to produce the narrowing of inter-individual variation in gene expression noted by Wittwer and colleagues (2004).

There is no definitive answer on the source of inter-individual variability in gene expression following exercise. The majority of work investigating this phenomenon has utilized human subjects and could be subject to sources of error not present in an animal model. The present work utilized inbred Sprague Dawley rats which represent a much more homogenous population than would be expected from random sampling of the human population. The use of muscle biopsies to take limited samples of muscle tissue for genetic analysis also represents a great source of error as different regions of the same biopsy can have drastically different expression profiles (Bakay et al., 2002). While this would certainly inflate intra-patient variability on repeated samples if consistent biopsies were not taken between individuals, inter-individual variability would also suffer due to regionalized differences in gene expression between different samples. In the present experiment small biopsies were not used and homogenization of whole muscle samples minimized this sampling variability.

Effects of exercise

Studies evaluating the significance of neurotrophins in the neuromuscular system typically use experimental approaches that apply exogenous neurotrophins to the tissue of interest. Unfortunately this methodology does not conform to the normal physiological domains of expression of the proteins of interest and the results of such work must be interpreted cautiously. As many neurotrophins display activity-dependent expression in various tissues, exercise could be an excellent in vivo model to study neurotrophins.

Voluntary wheel running is the most commonly used exercise model to determine activity-dependent expression of neurotrophins in various tissues (Adlard and Cotman, 2004; Adlard et al., 2004; Berchtold et al., 2002; Berchtold et al., 2001; Gomez-Pinilla et

al., 2002; Griesbach et al., 2004; Neeper et al., 1995; Oliff et al., 1998; Perreau et al., 2005; Russo-Neustadt et al., 2000; Tong et al., 2001; Vaynman et al., 2003; Vaynman et al., 2004a; Vaynman et al., 2004b; Widenfalk et al., 1999; Ying et al., 2005). This mode of exercise has been termed "sprint training" as animals can run at paces up to 50 $m \cdot min^{-1}$ and exceed their predicted maximum oxygen consumption estimates for very short intervals (Rodnick et al., 1989). Exercise at such a high intensity typically occurs in very short bouts and while the total distance covered by the animal can be great, often as high as 20 km per night (Widenfalk et al., 1999), the time spent running at or near maximum oxygen consumption is minimal.

This treatment has produced large increases in BDNF and trkB in the brain following as little as 3000 revolutions per night in controlled distance experiments and for up to five weeks in free running experiments (Adlard and Cotman, 2004; Adlard et al., 2004; Berchtold et al., 2002; Berchtold et al., 2001; Gomez-Pinilla et al., 2002; Neeper et al., 1995; Oliff et al., 1998; Perreau et al., 2005; Russo-Neustadt et al., 2000; Tong et al., 2001; Vaynman et al., 2003; Vaynman et al., 2004a; Vaynman et al., 2004b; Widenfalk et al., 1999). Increases in BDNF and trkB have been detected after as little as three days of VWR (Gomez-Pinilla et al., 2002; Oliff et al., 1998; Vaynman et al., 2003) however NT-4/5 has not been studied in this context in the central nervous system. The effect of voluntary exercise on NT-4/5 expression in skeletal muscle has also not been evaluated and requires further investigation.

Contrary to VWR, the metabolic requirements and adaptations to treadmill exercise have been rigorously tested however this model is rarely used to study activity-dependent expression of neurotrophins in skeletal muscle (Gomez-Pinilla et al., 2001).

This is surprising as the adaptations which occur in muscle following exercise are often more pronounced than those seen in voluntary wheel run animals (Jeneson et al., 2007). The primary advantage of a forced exercise protocol is the ability to control both the speed and grade of exercise and consequently have the animals maintain the time spent running at a higher intensity for longer than they would under their own volition. Alteration of the degree of inclination of the treadmill allows for greater recruitment of the lower limb musculature than would be expected in purely horizontal and voluntary wheel running (Gardiner et al., 1982). The soleus muscle is recruited heavily during treadmill locomotion even at slow speeds and shows little change in EMG with increased speed with no incline. When the work required of the animal is increased by applying an external load or increasing the grade of the treadmill recruitment of the lateral and medial gastrocnemius has been shown to increase (Gardiner et al., 1982). These results suggest that inclined treadmill running will result in greater activation of the gastrocnemius complex compared to activation levels during voluntary wheel running.

In the current study, treadmill exercise was used to facilitate the study of the medial gastrocnemius which has been neglected in the activity-dependent neurotrophin literature. One other study to date has utilized treadmill exercise to study neurotrophin expression in skeletal muscle but focused only on expression of BDNF and trkB in soleus. Gomez-Pinilla and colleagues (2001) studied changes in trkB and BDNF expression in soleus following five days of inclined treadmill exercise. While one exercise session was insufficient to increase expression of either BDNF or trkB there was a modest increase in expression after five exercise sessions. In the present study, a similar treadmill protocol failed to produce an increase in BDNF, trkB and NT-4/5. The duration

of the exercise intervention was ten days in the current work, twice as long as Gomez-Pinilla et al. (2001). It is possible that alterations in neurotrophin expression occur as an early adaptation to exercise and that the effect is resolved by the ten-day time point used in this study. Gomez-Pinilla et al. (2001) also utilized male rats whereas the present work was performed with female rats. Berchtold et al. (2001) found the increase in BDNF mRNA following exercise to be reduced in the absence of estrogen. This would suggest that any exercise-induced increase in BDNF expression would likely be greater in females than in males. Future work is required using shorter exercise protocols similar to Gomez-Pinilla et al. (2001) to determine if female rats have a heightened response to exercise with respect to BDNF expression. It is also possible that the speed of running and the degree of inclination of the treadmill could be responsible for the differing results of the present work with Gomez-Pinilla et al. (2001). The degree of inclination was higher in the present work (5% grade compared to 2.5%) and as a consequence the running speed of the animals was reduced to a maximum 20 $m \cdot min^{-1}$ to ensure the animals could complete each exercise session. While the effect of the total volume exercise on neurotrophin expression has been well documented in voluntary exercise models, the effect of exercise at different intensities has not been evaluated.

The brevity of the increase in neurotrophin expression with treadmill exercise has also been demonstrated with voluntary wheel running. Three days of voluntary wheel running lead to increased expression of both trkB and BDNF in soleus while only the increased expression of BDNF persisted (Gomez-Pinilla et al., 2002). While elevated BDNF mRNA and protein persisted over the seven-day exercise period, expression was peaked at three days of exercise. The current study cannot eliminate treadmill exercise as

a viable intervention to study muscle-derived neurotrophins as it is likely that a shorter training interval is required to observe changes in neurotrophin expression.

The lack of change in NT-4/5 following treadmill exercise agrees with data from Walker and Schon (1998). In an investigation into the relationship between NT-4/5 expression and oxidative dysfunction, aerobically trained individuals had NT-4/5 levels similar to sedentary controls. The authors theorized that NT-4/5 expression could be related to oxidative dysfunction and the generation of free radicals within skeletal muscle. As exercise can be a potent stimulus for the production of free radicals due to the increased energy requirements, it is possible that NT-4/5 expression is induced only when free radicals reach pathological levels. Accordingly, the energetic demands of the treadmill exercise in the current study may have been insufficient to invoke NT-4/5 expression through a similar mechanism.

Given the lack of change in expression of BDNF, NT-4/5 and TrkB with the current exercise protocol one could question whether the exercise was of sufficient volume or intensity to produce any training effect in the animals at all. In a separate experiment utilizing laser capture microdissection and RT-PCR to quantify gene expression in isolated motoneurons, GAPDH expression was found to be significantly increased 190% in the same animals used in the present work (unpublished laboratory data). These results indicate that the training intervention utilized in the current work was successful in inducing changes in mRNA encoding a protein integral to cellular metabolism in one component of the motor unit.

Effects of muscle-type

In the present study, inter-muscle differences in expression of BDNF, NT-4/5 and trkB could not be determined due to differences in expression of control genes. Inter-muscle comparisons represent an important research question that could help to determine the significance of the neurotrophin proteins and their receptors in skeletal muscle. In order to address this problem further work is required to identify suitable control genes. It is quite possible that an ideal control gene for evaluating differences in gene expression induced by exercise may be less than ideal for normalizing data for inter-muscle comparisons as was found in this study. Thus further investigation is needed to determine genes that have similar expression levels between different muscle groups and ideally whose expression is also resistant to exercise.

There is no conclusive data on muscle-type differences in neurotrophin expression. NT-4/5 was originally believed to be localized to type I fibers and was found to be expressed to a greater degree in SOL compared with MG (Funakoshi et al., 1995). Recent data contradicts this discovery and suggests that NT-4/5 is much higher in predominately fast-twitch muscles (Sakuma et al., 2001). While fiber-type specific differences in BDNF have not been investigated to the extent of NT-4/5 it is currently believed that BDNF is higher in slow twitch muscles as they contain a greater amount of satellite cells (Mousavi and Jasmin, 2006). How neurotrophins are involved or affected by the transformation of different muscles to meet their specific physiological demands is unclear at this time. If RT-PCR is to be used to further investigate this area, identification of appropriate control genes for data normalization is required.

Limitations

While RT-PCR is an extremely sensitive technique which allows for the precise detection of mRNAs of low abundance it lacks the ability to refine the precise localization of a particular mRNA. Due to the preparation of the experimental samples through manual homogenization it is possible that some of the detected mRNA was from cells other than skeletal muscle. While great care was taken during dissection to remove extra tissue such as fatty deposits, connective tissue and the sciatic nerve, such dissection could only be performed superficially. The contribution on non-muscle cells to the RT-PCR signal is indicated by the successful detection of β-actin, a non-muscle actin believed to be expressed by all cells other than skeletal muscle. If the exercise was of sufficient intensity to induce tissue damage the subsequent inflammation would attract both neurotrophils and macrophages which could have also contributed to detection of mRNA from non-muscle cells.

Conclusion

Ten days of forced treadmill exercise failed to elicit any significant differences in expression of BDNF, NT-4/5 and TrkB in skeletal muscle. The exercise parameters utilized in this study do not represent an effective model to study activity-dependent expression of BDNF, NT-4/5 and trkB in skeletal muscle when performed over ten days. Inter-muscle comparisons were prevented as normalization of expression was not possible due to non-equivalent expression of β-actin and GAPDH between soleus and medial gastrocnemius.

Future directions

The current study indicates that further work is required to understand the relationship of physical exercise and neurotrophin expression in skeletal muscle. While ten days of treadmill exercise did not elicit changes in BDNF, NT-4/5 and trkB in skeletal muscle it is possible that the effects of exercise occur at an earlier time point. Five days of treadmill exercise have been shown to increase BDNF (Gomez-Pinilla et al., 2001) however it remains unknown whether this mode of exercise will alter NT-4/5 expression.

The demonstration of non-equivalent expression of the control genes β-actin and GAPDH between MG and SOL indicates further investigation to determine appropriate control genes for inter-muscular comparisons. Comparisons of muscles of differing phenotypes could be important in determining the significance of muscle-derived neurotrophins however without an appropriate control for data normalization these experiments cannot be performed. Future work should focus on the expression of other housekeeping genes such as β2-microglobulin, cyclophilin and the common ribosomal 28s and 18s RNAs.

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