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**FACULTY OF GRADUATE AND
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M.Sc. (Neuroscience)

GRADE / DEGREE

Department of Cellular and Molecular Medicine

FACULTÉ, ÉCOLE, DÉPARTEMENT / FACULTY, SCHOOL, DEPARTMENT

The Role of Brain-Derived Neurotrophic Factor (BDNF) in Postnatal Muscle Development and Regeneration

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The Role of Brain-Derived Neurotrophic Factor (BDNF) in Postnatal Muscle Development and Regeneration

Charlene Clow

This thesis is submitted as a partial fulfillment of the M.Sc. program in Neuroscience

Department of Cellular and Molecular Medicine

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University of Ottawa

April 30th, 2010

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Your file *Votre référence*
ISBN: 978-0-494-73794-1
Our file *Notre référence*
ISBN: 978-0-494-73794-1

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Abstract

In adult skeletal muscle, brain-derived neurotrophic factor (BDNF) is expressed in a reserve population of myogenic progenitors known as satellite cells. Previous studies in our laboratory have shown that siRNA-mediated depletion of BDNF results in precocious differentiation of myoblasts in culture, suggesting a role in regulation of myogenic differentiation (J. Neuroscience, 2006). In order to functionally address the role of BDNF in muscle satellite cells and regeneration in vivo, we employed a Cre/Lox approach to generate a mouse in which BDNF is specifically depleted from skeletal muscle cells (BDNF^{MKO}). Cre recombinase expression was driven by the Myf5 promoter, resulting in BDNF depletion during early myogenic lineage determination. For comparative purposes, and to determine the specific role of muscle-derived BDNF, we also examined skeletal muscles of the complete BDNF^{-/-} mouse. In both animal models, expression of myosin heavy chain (MyHC) type IIB was decreased at the transcript and protein levels. In addition, we found decreased expression of the satellite cell marker Pax7 compared to control littermates. Because satellite cells are responsible for postnatal growth and repair of skeletal muscle, we next examined the satellite cell pool using primary cultures. BDNF^{MKO}-derived myoblasts exhibited abnormal differentiation, with delayed induction of several molecular markers of differentiation and decreased myotube size. These defects were rescued by the addition of exogenous BDNF. To determine whether in vivo regenerative capacity was compromised in the absence of muscle-BDNF we performed cardiotoxin-induced regeneration assays. Indeed, BDNF-depleted muscle showed delayed expression of several markers of regeneration following injury. Furthermore, we observed delayed appearance of newly regenerated fibers in the absence of muscle-BDNF. Together these findings suggest that muscle-derived BDNF plays at least two functions in skeletal muscle compartment; in promoting expression of MyHC IIB and in regulating myogenic differentiation during regeneration.

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List of Abbreviations

BCA: Bicinchonic acid

BDNF: Brain-derived neurotrophic factor

bHLH: Basic helix-loop-helix

BrdU: Bromodeoxyuridine

CNS: Central nervous system

CNTF: Ciliary-derived neurotrophic factor

CTL: Control

CTX: Cardiotoxin

DEPC: diethylpyrocarbonate

DIA: Diaphragm

DMD: Duchenne muscular dystrophy

DMEM: Dulbecco's modified Eagle's medium

DNA: Deoxyribonucleic acid

EDL: Extensor digitorum longus

ELISA: Enzyme-linked immunosorbant assay

embMyHC: Embryonic myosin heavy chain

FBS: Fetal bovine serum

FDB: Flexor digitorum longus

FGF: Fibroblast growth factor

GAPDH: glyceraldehydes-3-phosphate dehydrogenase

GAS: Gastrocnemius

GDNF: Glial cell derived neurotrophic factor

GFP: Green fluorescent protein

HGF: Hepatocyte growth factor

IGF: Insulin-like growth factors

MEF: Myocyte enhancer factor

MKO: Muscle knockout

MMP: Matrix metalloproteinase

MPC: Myogenic precursor cell

MRF: Myogenic regulatory factor

MSTN: Myostatin

Myf5: Myogenic factor 5

MyHC: Myosin heavy chain

MyoD: Myogenic determination factor

P: Postnatal day

NADE: Neurotrophin-associated cell death executor

NCAM: Neuronal cell adhesion molecule

NGF: Nerve growth factor

NMJ: Neuromuscular Junction

NRAGE: Neurotrophin-receptor interacting MAGE homologue

NRIF: Neurotrophin-receptor interacting factor

NT-3: Neurotrophin-3

NT-4/5: Neurotrophin-4/5

NT-6: Neurotrophin-6

NT-7: Neurotrophin-7

NTR: Neurotrophin Receptor

OCT: Optimal cutting temperature

PCR: Polyacrylamide chain reaction

RNA: Ribonucleic acid

RT: Reverse transcription

SC1: Schwann cell factor 1

SOL: Soleus

TA: Tibialis anterior

TNF: Tumour necrosis factor

TRAF: TNF-receptor associated factor

Trk: Tropomyosin related kinase

UTR: Untranslated region

VCAM: Vascular cell adhesion molecule

Acknowledgements

I would like to thank Dr. Bernard Jasmin for providing me with the opportunity to complete my Master's degree in his laboratory. I feel very fortunate to have been part of the Jasmin laboratory for the past 4 years. My experiences and training have been invaluable, and I am grateful for the opportunities that I gained as a graduate student. My experiences in the Jasmin lab have helped me to define my goals, and have provided me with the foundation I will need to be successful in future endeavours. I would like to thank all lab members for their support and guidance throughout my research project. In particular, I would like to thank John Lunde and Amanda Shaver for technical assistance, and Drs. Pedro Miura and Kambiz Mousavi for countless discussions that guided me through all aspects of my degree. I would also like to acknowledge the members of my advisory committee, Drs. Lynn Megeney, Stephen Gee and most recently Rashmi Kothary, who provided clear and thoughtful input and advice.

On a personal note, I owe this work to a number of people outside the lab who helped me balance myself for life outside the lab. The support of my parents has been an integral part of my success throughout my postsecondary education (and life in general). Thanks for teaching me to do what I love and that the rest will follow. Finally, I would like to thank my soon-to-be husband, Jeff Massier who has always been there for me no matter where I needed to be or what I needed to do to become the person I am today.

CHAPTER 1

Introduction

1. Introduction

1.1 The Neurotrophin Family

Neurotrophins are a highly homologous family of secreted proteins best known for their roles in regulating the survival, differentiation, growth, and death of neurons (Kalb, 2005; Oppenheim et al., 1991; Hamburger, 1984). Genetic analysis reveals that neurotrophins are highly conserved throughout evolution, and suggests that all neurotrophins were derived through successive duplications of a single ancestral gene (Hallbook, 1999; Lanave et al, 2007). The mammalian family of neurotrophins consists of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5). In lower vertebrates, NGF-like neurotrophins, NT-6 and NT-7 have also been identified (Gotz et al, 1994; Nilsson et al, 1998).

All neurotrophin genes contain multiple promoters, each flanking a downstream non-coding exon. These 5' non-coding exons are followed by a single 3' exon containing all of the information encoding the pre-propeptide (Fig. 1A; Suter et al, 1991; Seidah et al, 1996). Non-coding exons are spliced independently of one another to the coding exon in order to generate bipartite transcripts containing various 5' untranslated regions (UTRs) followed by a common coding sequence (Bertaux et al, 2005; Aid et al, 2007). As such, identical neurotrophin protein products are encoded by various mRNAs that differ only in the sequence of their UTRs (reviewed, Reichardt, 2006). The functional significance of multiple non-coding sequences is unclear, however, tissue-specific and activity dependent promoter usage suggest that the 5'UTR represents an important transcriptional and/or post-transcriptional regulatory region. Further complexity has been uncovered in the regulation of BDNF, where alternative polyadenylation

sites within the 3'UTR have been shown to produce distinct long and short transcripts which are differentially localized, and confer different functional roles within the central nervous system (An et al, 2008). These transcripts are likely regulated at the post-transcriptional level by trans-acting elements which bind differentially depending on the presence or absence of regulatory regions within the long and/or short 3'UTRs. These elements may include RNA binding protein target sites and/or micro-RNA consensus sequences.

The protein product of each neurotrophin gene includes a signal sequence and prodomain followed by the mature neurotrophin sequence (Fig. 1B; reviewed, Chao and Bothwell, 2002). Cleavage of pro-neurotrophins (MW 30-35 kDa) to generate the C-terminal mature protein (MW 12-13 kDa) can occur intracellularly; mediated by furin, a calcium-dependent serine protease (Bresnaha et al, 1990; Seidah et al, 1996, Mowla et al, 2001). Extracellular cleavage can be mediated by matrix metalloproteinases such as MMP3 and MMP7 (Lee et al, 2001), or the pericellular serine protease, plasmin (Pang et al, 2004; Gray and Ellis, 2008). Although early work suggested that pro-neurotrophins acted as dominant-negative signal antagonists, work in recent years has shown that un-cleaved pro-neurotrophins (pro-NGF and pro-BDNF) can confer signalling capabilities through binding to the low-affinity neurotrophin receptor, p75^{NTR}. When pro-neurotrophins bind to p75^{NTR} in a complex with sortillin, a member of the Vps10p-domain family of receptors, it results in the activation of apoptotic pathways and cell death (Lee et al, 2001; Nykjaer et al, 2003). Thus, in addition to the multiple transcriptional and post-transcriptional mechanisms governing neurotrophin expression, mature neurotrophin proteins can be post-translationally regulated by the levels of various proteases responsible for conversion of the pro-neurotrophins to mature neurotrophins.

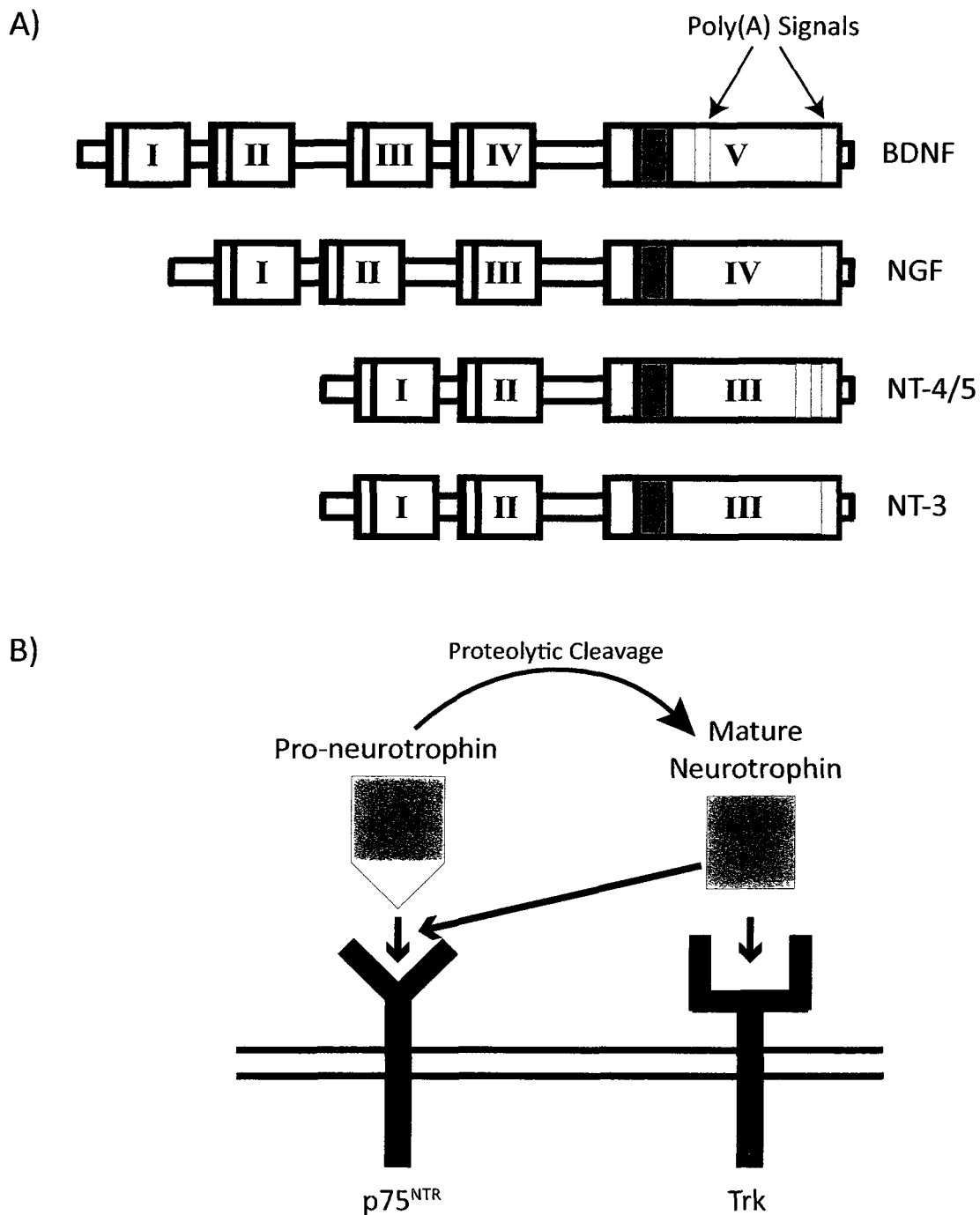


Figure 1. Schematic representation of neurotrophin gene structure and organization. A) White boxes represent untranslated regions. Upstream promoters are represented by yellow boxes. The pro-domain (pink) is followed by the mature neurotrophin protein coding sequence (red). 5' polyadenylation signals are represented by blue lines. B) Neurotrophins are produced as large precursor proteins known as pro-neurotrophins. This gene product must be proteolytically processed to form the mature neurotrophin protein. Mature neurotrophins can bind to high affinity Trk receptors and/or the low affinity p75 neurotrophin receptor (p75^{NTR}) to elicit downstream signal transduction. Uncleaved pro-neurotrophins are unable to bind Trk receptors, but can bind p75^{NTR} with high affinity.

1.2 Neurotrophin Receptors and Signal Transduction

Neurotrophins activate downstream signalling pathways by binding to one of two types of receptors; the tropomyosin related kinase receptors (Trk A, B, C), or the low-affinity neurotrophin receptor, p75^{NTR} (Klein et al, 1991; Bibel and Barde, 2000; Schweigreiter, R. 2006). Trk receptors exhibit ligand specificity and high-affinity binding properties. Specifically, NGF binds TrkA, BDNF and NT-4 bind TrkB, and NT-3 binds TrkC (Huang and Reichardt, 2003). Mature neurotrophins bind to their respective Trk receptors as non-covalently bound homodimers (reviewed; McDonald and Chao, 1995). This results in dimerization and transphosphorylation of cytoplasmic tyrosine kinases, and creates docking sites for adaptor molecules that ultimately promote survival and/or differentiation through activation of Ras/ERK, PI3/Akt or PLC- γ signalling cascades (Fig. 2A; Cunningham et al, 1997; Patapoutian and Reichardt, 2001; Arevalo and Wu, 2006). It is important to note that Trk receptors are activated specifically by mature and not the pro-neurotrophins (Lee et al, 2001) Thus, the levels of proteases that promote cleavage of pro-neurotrophins to their mature counterparts are also important regulators of Trk receptor activity (reviewed; Reichardt, 2006). Finally, alternative splicing of Trk receptors can result in the formation of receptor isoforms lacking kinase activity. These truncated isoforms are particularly abundant outside the nervous system, and were previously thought to act as scavengers or dominant-negative receptors (Middlemas et al, 1991; Barker et al, 1993; Eide et al, 1996). However, this hypothesis has been challenged by recent studies showing that truncated Trk receptors, particularly truncated TrkB (TrkB-T1), can confer functionally significant signal transduction; including calcium signalling in glial cells (Rose et al, 2003), and cell fate decisions in neural stem cells (Islam et al, 2009).

The low affinity neurotrophin receptor, p75^{NTR} belongs to the tumour necrosis factor (TNF) receptor super family, and binds all mature neurotrophins with similar (~10µM) affinity (Chao and Hempstead, 1995; Roderiguez-Tebar et al, 1990; Chao et al., 1986). Unlike Trk receptors, which bind exclusively to mature neurotrophins, p75^{NTR} has been shown to bind pro-neurotrophins, particularly pro-NGF and pro-BDNF, with high affinity (Nykjaer et al, 2004). Neurotrophin binding to p75^{NTR} mediates a complex array of signalling cascades that can promote survival, differentiation, growth or death depending on the cellular/physiological context (Fig. 2B; Arevalo and Wu, 2006, Nykjaer *et al.*, 2005; Barker, 2004). Although the intracellular domain of p75^{NTR} lacks intrinsic enzymatic activity, neurotrophin binding promotes signal transduction through activation of p75^{NTR}-associated adaptor proteins. These consist of pro-apoptotic proteins, including neurotrophin receptor interacting MAGE homologue (NRAGE), neurotrophin-associated cell death executor (NADE), TNF-receptor-associated factors 2 and 6 (TRAF2 and TRAF6) and neurotrophin-receptor interacting factor (NRIF) (Salehi et al, 2000; Mukai et al, 2000; Ye et al, 1999; Khursigara et al, 1999; Casademunt et al, 1999). Additionally, p75^{NTR} can regulate cell cycle progression through Schwann cell factor 1 (SC1) mediated cyclin E repression (Chittka et al, 2004), cell growth through activation of RhoA (Yamashita et al, 2003), and cell survival through activation of NF-κB signalling (Hamanoue et al, 1999). Finally, ligand binding to p75^{NTR} can result in sphingomyelin hydrolysis, which results in generation of ceramide (Dobrowsky et al, 1994). Ceramide can promote both apoptotic and prosurvival pathways (Muller et al, 1998; DeFreitas et al, 2001; Song and Posse de Chaves, 2003). Thus, cellular outcome depends on the presence/absence of co-receptors and proteolytic enzymes responsible for conversion of pro-neurotrophins to their mature form, as well as stoichiometric quantities of neurotrophin family members.

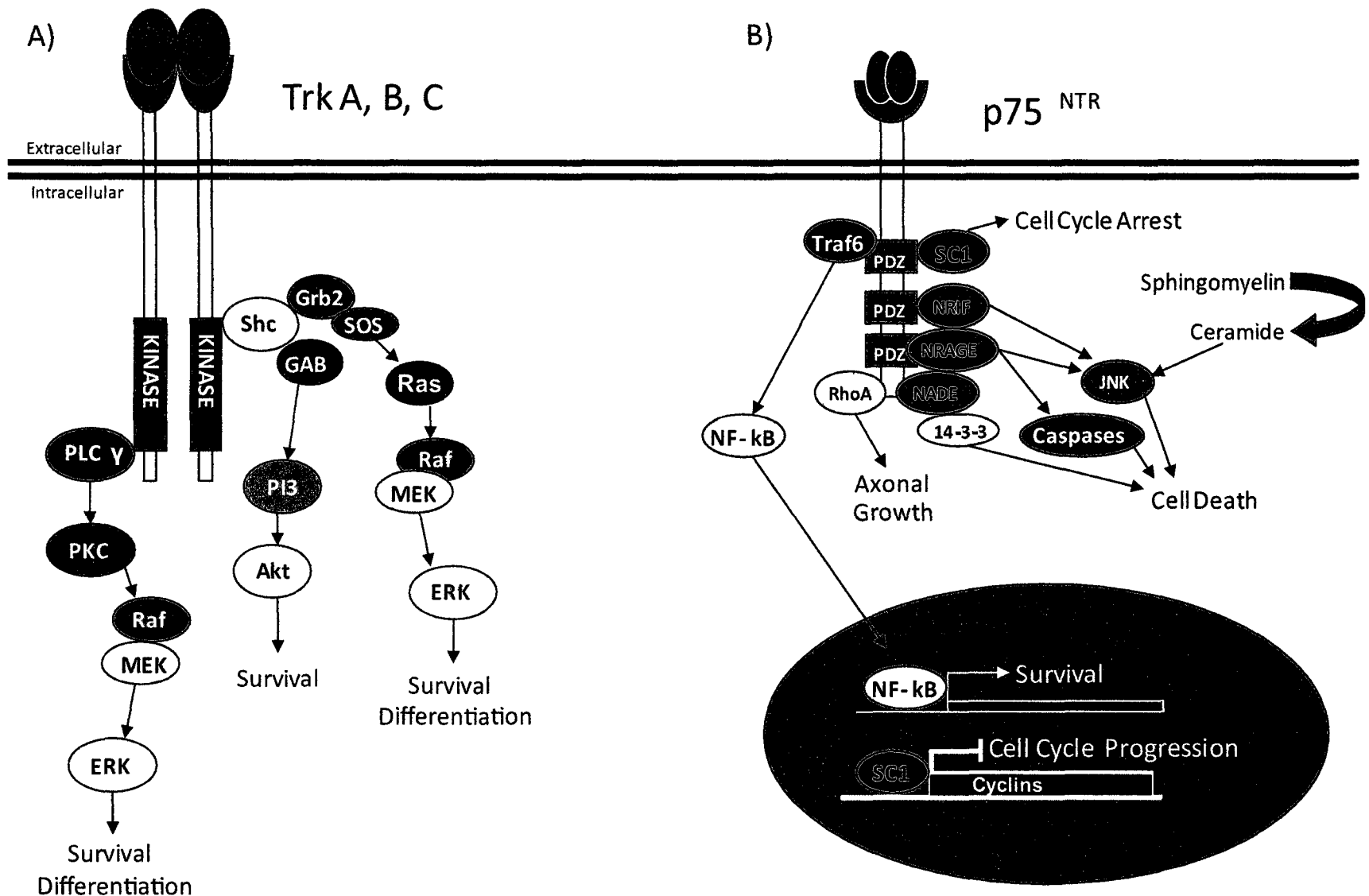


Figure 2. Schematic representation of neurotrophin-mediated signal transduction pathways. A) Tropomyosin related kinase receptors (Trk A, TrkB and TrkC) promote survival and differentiation through activation of Ras, phosphatidyl inositol 3 (PI3)-kinase, phospholipase C-γ1 pathways. B) Activation of p75^{NTR} results in activation of more complex signalling cascades that can promote survival, growth, cell cycle arrest, or death depending on the cellular context. (Adapted from Arevalo and Wu, 2006).

1.3 The Neurotrophic Hypothesis

Neurotrophins are expressed throughout the central nervous system (CNS), where they regulate a number of cellular processes in neuronal and glial cells (reviewed; Schinder and Poo, 2000; Woo and Lu, 2006). Furthermore, several groups have demonstrated that neurotrophins and their corresponding receptors are highly expressed in non-neuronal cell populations throughout the periphery (Table 1, Escandon et al, 1994; Foster et al, 1994; Ip et al, 1994; Donovan et al, 1995; Yamamoto *et al*, 1996; Ladiwala et al, 1998; Heinrich et al, 1999; Labouyrie et al, 1999; Miknyoczki et al, 1999; Nakahashi et al, 2000; Barouch et al, 2001; Hikawa et al, 2002; Sheard *et al.*, 2002; Lai and Ip, 2003; Vega et al, 2003; Van't Veer et al, 2009). The most widely accepted model of neurotrophin function across tissue systems, *the neurotrophic hypothesis*, was proposed by Hamburger and colleagues following the observation that neuronal survival depends on the presence of the target tissue (Hamburger, 1934). These studies demonstrated that removal of the limb bud at early stages of chick development results in complete abrogation of spinal motor neurons. In addition, they showed that the number of innervating motor neurons could be altered by adjusting the size of the target organ (by transplanting or removing limb bud), such that larger targets could support a higher number of neurons (Hamburger and Levi-Montalcini, 1949; Levi-Montalcini and Hamburger, 1951; Levi-Montalcini and Hamburger, 1953). The neurotrophic hypothesis ensued, suggesting that targets of innervation secrete a limited quantity of survival factor (neurotrophic factor) in order to ensure the balance between the size of the target organ, and the number of (surviving) innervating neurons (Purves, 1988; Davies, 1996). Thus, although motor neurons are generated in excess during early development, only those neurons which reach the target tissue and receive neurotrophic support would survive; with all others dying during the period of naturally

occurring cell death (Fig. 3; reviewed, Oppenheim, 1991). Nerve growth factor, and subsequently the neurotrophin family of proteins, was discovered in the initial search for neurotrophic factors (Cohen et al, 1954; Cohen, 1960; Shooter, 2001). Ensuing studies showed that exogenous application of neurotrophins, BDNF, NT-3 and NT-4, could rescue a percentage of neurons that would normally die within the period of naturally occurring cell death. Moreover, a number of other secreted factors were shown to support neuronal survival and differentiation, including ciliary derived neurotrophic factor (CNTF), glial cell derived neurotrophic factor (GDNF), as well as classical growth factors (IGF I, IGF-II, TGF-B). Combined administration of neurotrophins with these other neurotrophic factors were shown to have a more pronounced rescue effect than application of individual neurotrophins (Arakawa et al, 1990; Hughes et al, 1993; Zurn et al, 1996; Mousavi et al, 2004).

Following the pioneering studies of Hamburger and Levi-Montalcini, several groups demonstrated that neurotrophins can be secreted pre- or postsynaptically, and can function as autocrine and/or paracrine signalling molecules. Consistent with the classical neurotrophic hypothesis, a number of groups showed that postsynaptically secreted neurotrophins can be internalized and retrogradely transported to the cell body in order to influence nuclear events controlling neuronal plasticity and survival (Riccio et al, 1997; Wang and Poo, 1997; Mufson et al, 1999). For example, NT-4 overexpression within the post-synaptic muscle cell results in synaptic potentiation in *Xenopus* muscle-nerve co-cultures (Wang and Poo, 1997). Evidence for pre-synaptic neurotrophin secretion was elegantly demonstrated in studies of the visual system, which showed that exogenously supplied NT-3 is internalized by retinal ganglion cells, anterogradely transported to axon terminals and released for uptake by second-order neurons (Bartheld et al, 1996). Finally, these studies showed that neuronal activity can regulate

Table 1. Tissue distribution of neurotrophins and neurotrophin receptors.

Tissue	NGF	BDNF	NT-3	NT-4/5	TrkA	TrkB	TrkC	p75 ^{NTR}
Brain	+	+	+	+	+	+	+	+
Sciatic Nerve	+	+	+	+/-	+/-	+	+	+
Spinal Cord	+	+	+	+	+	+	+	+
Oligodendrocytes	+	+	+	+	-	+	+	+
Schwann Cells	+	+	+	+	-	+	+	+
Heart	+	+	+	+/-	-	+/-	+	+
Endothelial cells	+/-	+	+	+/-	+	+	+	-
Thymus	+	+	+	+	+	+	-	+
Macrophages	+	+	-	+	+	+	+/-	-
Mast cells	+	+	+	-	+	+	+	-
Kidney	+	-	+	-	+	+	+	+
Liver	-	+	+	-	-	-	-	+
Pancreas	+	+/-	+	+	+	+	+	-
Spleen	+	+	+	-	+/-	+	-/+	+
Lung	+	+	+	+	+	+/-	+	+
Skeletal Muscle	+	+	+	+	+/-	+/-	+	+

+, expression reported; -, no expression reported, +/-, contradictory results reported

(Results compiled from studies by Escandon et al, 1994; Foster et al, 1994; Ip et al, 1994; Donovan et al, 1995; Yamamoto et al, 1996; Ladiwala et al, 1998; Heinrich et al, 1999; Labouyrie et al, 1999; Miknyoczki et al, 1999; Nakahashi et al, 2000; Barouch et al, 2001; Hikawa et al, 2002; Sheard et al, 2002; Lai and Ip, 2003; Vega et al, 2003; Van'tVeer et al, 2009)

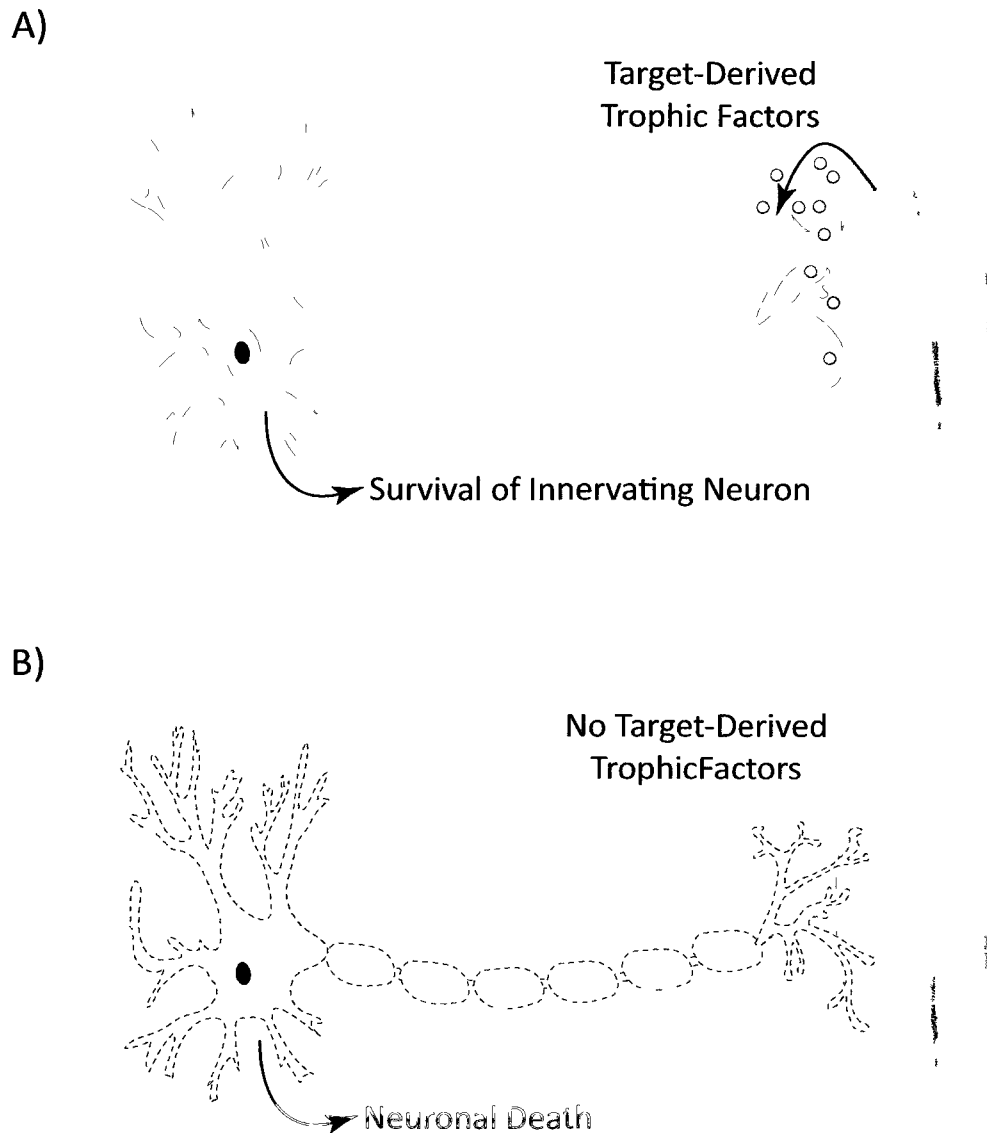


Figure 3. Neurotrophic Hypothesis of Neuronal Survival. A) Schematic representation of motor neuron survival in response to the presence of target (muscle)-derived trophic factors (\circ). B) In the absence of trophic support, excess motor neurons are eliminated during the period of naturally occurring cell death (adapted from Davies, 1996)

neurotrophin synthesis, secretion and signal transduction (Schinder and Poo, 2000). Activity dependent neurotrophin expression was first demonstrated in hippocampal neurons, where treatment with kainic acid, a glutamate receptor agonist, results in the dramatic upregulation of NGF and BDNF within hippocampal neuron cultures *in vitro* (Zafra et al, 1990), and in the CA1 layer of the hippocampus *in vivo* (Yan et al, 1997).

1.4 Neurotrophin Knockout Mice

Ablation of neurotrophins and/or their receptors is postnatal lethal, with the exception of NT-4/5 and p75^{NTR} knockout animals, which persist to adulthood (Klein et al, 1993; Crowley, 1994; Ernfors et al, 1994a; Ernfors et al, 1994b; Jones et al, 1994; Liu et al, 1995). The predominant phenotypes presented in these mice involve sensory neuronal defects, and surprisingly, ablation of individual neurotrophins does not result in dramatic motor neuron cell death (Conover et al, 1995). Motor deficits were only observed in NT-3 knockout animals, where development of muscle spindles and gamma motor neurons is impaired, and TrkB knockouts, which have neuronal deficiencies in both the central and peripheral nervous systems (Klein et al, 1993; Kucera et al, 1995; Patel et al, 2003). Instead, neurotrophin knockout mice often exhibit developmental abnormalities distinct from effects on neuronal survival (Table 2; Donovan et al, 1996; Tessarollo et al, 1997; Capsoni et al, 2000; Donovan et al, 2000; Garcia-Suarez et al, 2000; Kernie et al, 2000; Ruberti et al, 2000; Belluardo et al, 2001; Garcia-Suarez et al, 2002; Carrasco and English, 2003; Wooley et al, 2003; Unger et al, 2007; Garcia-Suarez et al, 2009; Kendal et al, 2009). Non-neuronal defects have been observed across a number of peripheral tissue systems, including cardiovascular, endocrine, reproductive, immune, and

skeletal musculature (reviewed, Tessarollo, 1998; Pitts et al, 2006). These studies suggest that neurotrophins, in addition to regulating neuronal survival, can signal in an autocrine or paracrine manner to influence resident cell populations in the periphery (reviewed; Sariola, 2001; Chevrel *et al.*, 2006). Thus, although the neurotrophic hypothesis has survived over 50 years of scrutiny, accumulating evidence suggests that neurotrophins may play a more widespread physiological role in non-neuronal tissue than originally believed.

1.5 Neurotrophins and Skeletal Muscle

Skeletal muscle is a principal supplier of neurotrophic support in the peripheral nervous system. As such, the majority of research has focused on the role of muscle-derived neurotrophin proteins in regulating motor neuron survival and function in the neuromuscular system. Although such research has confirmed that skeletal muscle-derived neurotrophins enhance motor neuron survival (Gonzalez *et al.*, 1997; Sendtner *et al.*, 1992) it has also revealed a role for neurotrophins in skeletal muscle development and function (Seidl *et al.*, 1998). Key to this discovery was the demonstration that neurotrophins and their receptors are indeed expressed in the skeletal muscle (Ip *et al.*, 2001). Several groups have also shown that skeletal muscle neurotrophin expression is dependent on the developmental context, and can be rapidly modified in response to injury or disease (Table 3; Furukawa *et al.*, 1979; Zhao *et al.*, 1991; Meyer *et al.*, 1992; Funakoshi *et al.*, 1993; Fernyhough *et al.*, 1995; Griesbeck *et al.*, 1995; Fernyhough *et al.*, 1996; Ihara *et al.*, 1996; Walker and Schon, 1998; Gomez-Pinilla *et al.*, 2001; Gomez-Pinilla *et al.*, 2002; Sakuma *et al.*, 2002; Toti *et al.*, 2003; Omura, 2005).

Table 2. Neurotrophin Knockout Mice

Knockout Mouse	Phenotype	Reference
NGF	Neuronal Loss: Sensory and Sympathetic Ganglia Spleen Cell Death Skeletal Muscle Dystrophy	Crowley et al, 1994 Ruberti et al, 2000 Capsoni et al, 2000
NT-3	Neuron loss: Sensory and Sympathetic Ganglia, α -motor neurons Defective muscle-spindle development Abnormal cardiovascular development Disassembly of the neuromuscular junction	Ernfors et al, 1994; Kucera et al, 1995 Ernfors et al, 1994 Donovan et al, 1996 Wooley et al, 2003
NT-4/5	Neuronal loss: Sensory Ganglia Muscle fiber-type switching Disassembly of the neuromuscular junction	Liu et al, 1995 Carrasco and English, 2003 Belluardo et al, 2001
BDNF	Neuronal loss: Sensory and Sympathetic Ganglia Hyperphagia and Obesity Abnormal cardiovascular development	Ernfors et al, 1994; Liu et al, 1995 Kernie et al, 2000; Unger et al, 2007 Donovan et al, 2000
TrkA	Neuronal Loss: Sympathetic and Sensory Ganglia Abnormal development of the thymus	Smeyne et al, 1994 Garcia-Suarez et al, 2000
TrkB	Neuronal Loss: Sensory, Sympathetic ganglia, spinal motor neurons Disassembly of the neuromuscular junction Abnormal lung development Abnormal development of the thymus	Klein et al, 1993 Gonzalez et al, 1999 Garcia-Suarez et al, 2009 Garcia-Suarez et al, 2002
TrkC	Neuronal Loss: Sensory Ganglia Defective muscle-spindle development Abnormal cardiac development	Klein et al, 1994 Klein et al, 1994 Tessarollo et al, 1997
p75NTR	Neuronal Loss: Sensory Ganglia Abnormal myofibroblast proliferation and fibrosis	Lee et al, 1992 Kendal et al, 2009

Table 3. Neurotrophins in skeletal muscle under physiological and pathophysiological conditions

Condition	Modification	Reference	
Exercise	↑BDNF	Gomez-Pinilla et al, 2001	
	↑NT-3	Gomez-Pinilla et al, 2001	
	↑TrkB	Gomez-Pinilla et al, 2002	
Denervation	↑NGF	Funakoshi et al, 1993	
	↑BDNF	Meyer et al, 1992; Griesbeck et al, 1995	
	↓NT-3	Funakoshi et al, 1993; Griesbeck et al, 1995	
	↓NT-4/5	Funakoshi et al, 1993; Griesbeck et al, 1995	
Diabetes	↓NGF	Fernyhough et al, 1995	
	↓NT-3	Ihara et al, 1996	
	↑BDNF	Fernyhough et al, 1996	
Myopathies			
	- Dystrophin deficient	↑NGF	Furukawa et al, 1979; Toti et al, 2003
	- Dystrophin deficient	↑p75 ^{NTR}	Zhao et al, 1991
	- Laminin deficient	↓NT-4/5	Sakuma et al, 2002
	- Mitochondrial	↓NT-4/5	Walker and Schon, 1998

↑, increased expression; ↓decreased expression

All neurotrophins are highly expressed during embryonic muscle development, with downregulation in early postnatal life (Griesbeck et al, 1995). In adult skeletal muscle, NT-4/5 is the most readily detected neurotrophin, followed by BDNF, NT-3 and NGF (Griesbeck et al, 1995; reviewed, Pitts et al, 2006). Interestingly, Trk receptor expression appears to be confined to embryonic muscle development (Ip et al., 2001), while p75^{NTR} is expressed at low levels throughout embryonic and post-natal muscle development (Yamamoto et al., 1996; Ernfors et al., 1988).

Although the majority of studies continue to focus on the neuronal deficits associated with neurotrophin depletion, a small number of groups have investigated the more subtle effects of neurotrophin depletion on skeletal muscle. Indeed, these studies have found that neurotrophin knockout mice often exhibit neuromuscular abnormalities distinct from effects on motor neuron survival. These include abnormal muscle spindle formation and loss of α -motor neurons in NT-3^{-/-} mice, dystrophic muscle pathology in NGF-depleted muscle, and neuromuscular junction (NMJ) disassembly and abnormal fiber-type transformation in the NT-4/5^{-/-} mouse (Table 2; Ernfors et al, 1994a; Capsoni et al, 2000; Belluardo et al, 2001; Carrasco and English, 2003). These findings, in conjunction with the tissue distribution of neurotrophins and their receptors have led to a number of new questions surrounding the functional significance of neurotrophins in skeletal muscle.

1.6 Skeletal Muscle Development

The majority of skeletal muscle is derived from progenitor cells originating in the somites. Somites arise from segmentation of the paraxial mesoderm, which is aligned along

either side of the neural tube during early embryonic development. Somites differentiate to form specialized regions in response to cell fate regulators (reviewed; Buckingham and Vincent, 2009). The ventral somites become the sclerotome, which gives rise to cartilage and bone. The dorsal somites differentiate to form dermomyotome, which gives rise to the dermis and skeletal muscles of the body and limbs. The specification of muscle progenitors within the dermomyotome depends on signals that emanate from neighbouring tissues such as the notochord, neural tube, and ectoderm (Buckingham, 1992).

The paired-box homeodomain (Pax) genes are well-known transcriptional regulators of organogenesis and cellular specification throughout embryonic development (Mansouri et al, 1994; Kawakami et al, 1997; Seale 2000). Two closely related Pax genes, Pax3 and Pax7, have been shown to play essential roles in myogenic programming (Seale, 2000; Buckingham and Relaix, 2007). Pax3 is expressed by presomitic mesoderm, and later in myogenic precursor cells (MPCs) which give rise to hypaxial muscles. Pax3 appears to function upstream of the early myogenic determination factor MyoD, and in the absence of Pax3, hypaxial muscles are completely absent (Williams and Ordahl, 1994; Tajbakhsh et al, 1997). Pax3 has also been shown to play an important role in MPC migration, through transcriptional regulation of the c-met receptor tyrosine kinase expression (Epstein et al, 1996). c-met activation by hepatocyte growth factor (HGF) is necessary for migration of myogenic precursors into the limb bud, and ablation of either c-met or HGF limb muscles are completely absent (Bladt et al, 1995; Schmidt et al, 1995). Importantly, these mutations do not affect the initial establishment of a myogenic precursor cell pool, however, delamination and migration is defective, leaving MPCs in the somatic compartment (reviewed; Birchmeier and Brohmann, 2000). Subsequent myogenesis depends on Pax3/7 MPCs which contribute to embryonic and neonatal development, and later to

establishment of a reserve population of MPCs in adult skeletal muscle (Gros et al, 2005; Kassari-Duchossoy et al. 2005; Relaix et al. 2005; *details Section 1.8*).

Embryonic muscle development involves 2 distinct waves of MPC delamination and migration from the dermamyotome to the developing limb bud. The first wave of MPCs originates along the dorsomedial lip, and migrates to the limb bud where they begin to express myogenic determination genes, proliferate, and fuse to form multinucleated primary muscle fibers. These primary myofibers function as a scaffold for secondary fiber formation. The second wave of MPCs originates from all borders of the dermomyotome, and becomes incorporated among the pre-existing myofibers within the developing limb bud (Kahane et al, 2002). As myofibers mature, they become innervated and vascularised, allowing regulated expression of muscle regulatory genes and contractile proteins.

1.8 Muscle Regeneration and Satellite Cells

Adult skeletal muscle is composed of post-mitotic, terminally differentiated myofibers. As such, postnatal growth and repair depends almost exclusively on a resident pool of muscle stem cells known as satellite cells (reviewed; Morgan and Partridge, 2003; Charge and Rudnicki, 2004). In healthy adult skeletal muscle, satellite cells are mitotically quiescent; sequestered between the basement membrane and sarcolemma of myofibers (Schultz et al, 1978; Lipton and Schultz, 1979). In response to injury however, these cells become activated; triggering rapid proliferation and differentiation to restore the damaged myofiber (Bischoff, 1975; Snow, 1978; Lipton and Schultz, 1979). Although satellite cells represent only 2-5% of myonuclei in adult skeletal muscle, when activated they have the ability to generate sufficient progeny to replace

entire muscle fibers (Zammit et al, 2002; Collins et al, 2005). Strikingly, as few as seven satellite cells have been shown to give rise >100 new myofibers (Collins et al, 2005). Expression profiling studies have revealed a number of molecular markers of satellite cells, including cell surface markers (α 7-integrin, Syndecan-3 and Syndecan-4), adhesion molecules (m-cadherin, vascular cell adhesion molecule [VCAM], and neuronal cell adhesion molecule [NCAM], and myogenic transcription factors such as Pax7 (reviewed; Tedesco et al, 2010).

While the majority of proliferating satellite cells differentiate and fuse to contribute new myonuclei to the regenerating fiber, a minority of these cells will reconstitute the undifferentiated satellite cell pool in order to support future rounds of degeneration and repair (Zammit et al, 2004; Collins et al, 2005). Maintenance of the satellite cell pool is essential for postnatal muscle growth and repair, and has been shown to depend on self-renewal of existing satellite cells. Direct evidence for self-renewal was shown by grafting GFP-labelled satellite cells into muscles of immunodeficient mdx mice, the dystrophin-deficient mouse model of Duchenne muscular dystrophy. Grafted satellite cells were not only capable of muscle repair, but also of repopulating the satellite cell pool as demonstrated by retrieval of GFP+; Pax7+ cells from the satellite cell position following single-fiber isolation (Collins et al, 2005; Montarras et al, 2005). A number of mechanisms for self-renewal have been suggested, including de-differentiation, asymmetric division, and repopulation by non-muscle stem cells (reviewed; Collins, 2006). Whatever the mechanism, self-renewal is imperative for maintenance of the satellite cell pool throughout postnatal life, and depletion of the satellite cell pool severely compromises the regenerative capacity of muscles in aging and disease. Thus, it becomes clear that regulation of the satellite cell number and functionality is imperative for efficient postnatal myogenesis, and mis-regulation of either process could adversely affect the regenerative process.

1.9 Molecular Regulation of Myogenesis

At the molecular level, myogenesis depends on the regulated expression of basic helix-loop-helix (bHLH) transcription factors, including Pax genes and myogenic regulatory factor (MRF) family members (Fig. 4, Rudnicki et al, 1992; Hasty et al, 1993; Nabeshima et al, 1993; Megeney et al, 1996; Seale et al, 2000; Tajbakhsh and Buckingham, 2000). These factors control determination and differentiation of cells that will form skeletal muscle.

While embryonic MPC specification depends on expression of both Pax3 and Pax7, postnatal myogenic programming of adult satellite cells express primarily Pax7 (see *Section 1.6*; Seale, 2000; Buckingham and Relaix; 2007). The Pax7^{-/-} mouse undergoes normal embryonic development, but lacks satellite cells necessary for postnatal growth and regeneration (Seale et al, 2000; Oustanina et al, 2004). Several years of research has since uncovered a critical role for Pax7 in specification and survival of muscle satellite cells (Seale, 2000; Relaix et al, 2005; Kuang et al, 2006; Lepper et al, 2009). Although a number of Pax target genes have been implicated in directing precursor cell fate towards the myogenic lineage, both Pax3 and Pax7 have been shown to positively regulate myogenic lineage progression through activation of MRF genes (Maroto et al, 1997; Tajbakhsh et al, 1997; Bajard et al, 2006; McKinnell et al, 2008).

The MRF family of bHLH transcription factors includes myogenic determination factor 1 (MyoD), myogenic factor 5 (Myf5), myogenic regulatory factor 4 (MRF4) and myogenin. These proteins activate transcription of regulatory and structural muscle genes by binding to a DNA consensus sequence (CANNTG) known as the E box, which is present in the promoter of numerous muscle-specific genes. MRFs have distinct but overlapping functions, with distinct temporal expression patterns throughout embryonic and postnatal myogenesis. Myf5 and MyoD

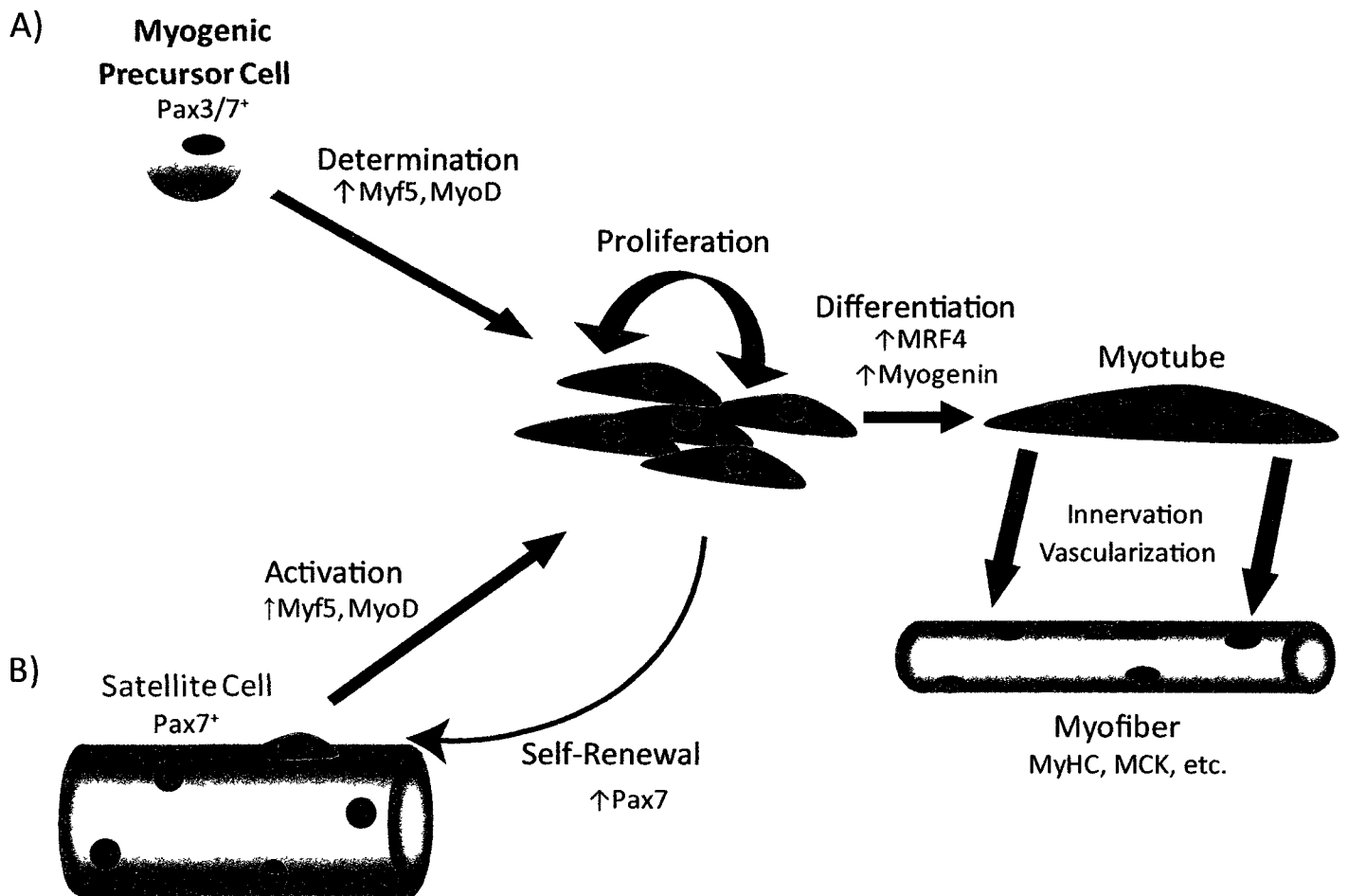


Figure 4. Myogenesis in the embryo and adult. A) Embryonic muscle development is controlled by the expression of Pax3 and myogenic regulatory factors (MRFs). Myogenic precursor cell commitment to the myogenic lineage depends on upregulation of ‘early’ MRFs, MyoD and Myf5. Determined myoblasts undergo several rounds of proliferation before upregulating ‘late’ MRFs, MRF4 and Myogenin, to become terminally differentiated myocytes. Myocytes fuse to form multinucleated myotubes which express developmental myosin heavy chain (MyHC) contractile proteins. As myotubes mature they become innervated and vascularized to form contractile muscle fibers expressing mature MyHC isoforms. B) Postnatal myogenesis depends on a reserve population of myogenic progenitor cells known as satellite cells. In healthy animals, satellite cells are mitotically quiescent, expressing Pax7, and situated between the basement membrane and fiber sarcolemma. In response to injury, satellite cells become activated, upregulate MyoD and Myf5, proliferate and undergo myogenic differentiation in a manner that recapitulates, in many ways, embryonic muscle development. While the majority of satellite cells differentiate and fuse to repair damaged muscle fibers, others will continue to express Pax7 and return to quiescence in order to maintain the satellite cell pool for future rounds of damage and repair.

are involved in myogenic lineage determination, as demonstrated by the complete loss of skeletal muscle in MyoD; Myf5 double knockout animals (Rudnicki et al, 1993). Mice lacking either of these genes alone however, do not exhibit a major muscle phenotype, suggesting that these genes can compensate for one another (Braun et al, 1992; Rudnicki et al, 1992). Myf5 expression is initially detected very early in development, where it has been shown to regulate myoblast proliferation and determination. MyoD expression follows Myf5, and is required for differentiation of myoblasts. Although satellite cells are present in the MyoD^{-/-} mouse, they cannot undergo terminal differentiation (Megency et al, 1996; Yablonka-Reuveni et al, 1999). Instead, MPCs accumulate in the regenerating myofiber, but myogenic lineage progression does not occur to allow proper repair of damaged fibers. MyoD has been described as a master regulator of differentiation-linked genes, and forced expression of MyoD in non-muscle cell lines is sufficient to induce cell fate changes to the myogenic lineage (Tapscott, 2005)

Myogenin and MRF4 are necessary for myogenic differentiation and formation of myotubes. Myogenin knockout mice exhibit a severe deficiency in skeletal muscle at birth, due to deficits in myoblast differentiation (Hasty et al, 1993; Nabeshima et al, 1993). Interestingly, MyoD-expressing myoblasts are present in these animals, and primary myofibers develop normally. Only secondary myofiber formation is unable to proceed, suggesting that myogenin is necessary for terminal differentiation of myoblasts (Venuti et al, 1995). More recently, myogenin has also been shown to negatively regulate Pax7 expression (Olguin, 2007). MRF4 knockout mice exhibit a range of phenotypes, and it has been suggested that MRF plays an early role in determination of a subset of myogenic precursors (Kassar-Duchossoy et al, 2004), as well as later roles in myogenic differentiation (Zhang et al, 1995; Rawls et al, 1998). Importantly, targeting of either myogenin or MRF4 into the Myf5^{-/-}; MyoD^{-/-} double knockout mouse does

not rescue muscle formation, suggesting that these genes function primarily as differentiation factors for myogenic precursor cells (Wang and Jaenisch, 1997).

MRFs cooperate with a second family of transcription factors, myocyte enhancer factor 2 (MEF2) to regulate expression of a vast array of target genes, including those involved in cell cycle and growth control, neuromuscular synapse formation, cytoskeletal and contractile apparatus, ion transport and calcium homeostasis, and cellular stress response (Blais et al, 2005).

1.10 Secreted Factors Regulating Satellite Cell Function

In addition to intrinsic (cellular, molecular, and genetic) regulators of myogenic differentiation, extrinsic (environmental) cues, such as secreted growth factors, inflammatory cytokines, and neuronal factors have been shown to regulate satellite cell function. The influence of these 'external' signals (either permissive or repressive) is critical for the proper spatial and temporal activation of a number of regulatory genes during muscle development and regeneration. The most basic example of this phenomenon is myogenic differentiation of cultured myoblasts, which occurs in response to changes in culture conditions, such as growth factor withdrawal (reviewed, Florini et al, 1996). Further evidence for the importance of extrinsic factors in regulating of satellite cell function has been elegantly demonstrated in a series of muscle aging studies. Skeletal muscle regenerative capacity is severely impaired with age, resulting in inefficient repair of already-weakened muscle fibers. Cross-transplantation of aged (24 mo) skeletal muscle into young (4mo) hosts results in the regeneration of fibers that are similar in mass and force-generating capacity than young muscles grafted into the same hosts (Carlson et al, 1999). Conversely, young muscles grafted into old hosts showed impaired

regeneration. Thus, decreased regenerative potential appeared to depend predominantly on the age of the host environment, rather than the age of the donor muscle. More recent studies by Conboy and colleagues (2005) have used heterochromatic parabiotic pairings (ie. shared circulatory system) to expose aged skeletal muscle to young circulatory factors (Conboy et al, 2005). Here, exposure to young circulatory factors was sufficient to restore the regenerative potential of aged skeletal muscle to levels seen in young animals following freeze-injury (Conboy et al, 2005). Again, young muscle displayed inefficient progenitor cell activity and reduced regenerative potential upon exposure to aged circulatory factors, supporting the hypothesis that host environment is of primary importance to regenerative processes.

A number of secreted factors have been shown to regulate various stages of postnatal myogenesis in response to muscle damage. These include positive regulators of myogenesis, such as hepatocyte growth factor (HGF) and fibroblast growth factor (FGF), which have both been shown to promote satellite cell activation and proliferation (Allen and Boxholm, 1989; Allen et al, 1995; Johnson and Allen, 1995; Tatsumi et al, 1998; Miller et al, 2000), as well as insulin-like growth factors (IGFs), which can promote proliferation, differentiation and survival of satellite cells (Chakravarthy et al, 2000; Wilson et al, 2003; Latres et al, 2005; Wilson et al, 2006). Conversely, negative regulators of satellite cell activation, proliferation and differentiation have been characterized, including transforming growth factor β (TGF- β) family members and inflammatory cytokines such as IL-6. The best-characterized TGF- β family member in muscle is myostatin (MSTN). MSTN prevents myogenic lineage progression by inhibiting the transcriptional activity of MyoD (Thomas et al, 2000). In addition, MSTN can promote satellite cell quiescence by inhibiting entry to the cell cycle, and promoting degradation of cyclin D (Yang et al, 2007). Finally, it is important to note that in addition to their ability to

promote activation, proliferation and differentiation of satellite cells, a number of secreted factors appear to function as chemo-attractants, able to recruit satellite cells from uninjured areas of the muscle (Schultz et al, 1985; Watt et al, 1987).

1.11 BDNF in Skeletal Muscle

Recent studies in our laboratory have implicated the brain-derived neurotrophic factor (BDNF) in skeletal muscle differentiation. Specifically, we showed that BDNF is highly expressed in muscle satellite cells, and that depletion of BDNF, using siRNAs targeting the 5' coding exon, results in precocious differentiation of rat L6 myoblasts in culture (Mousavi and Jasmin, 2006). This suggests a role for BDNF in regulation of myoblast differentiation, and is in accordance with reports of elevated BDNF expression during embryonic development, where myoblasts are abundant (Griesbeck et al, 1995). Interestingly, other studies have shown that muscle-BDNF expression is rapidly upregulated following muscle injury (Griesbeck et al 1995; Funakoshi et al, 1993; Meyer et al, 1992); a situation where satellite cell activation and proliferation occurs in order to repair the damaged muscle fiber. Finally, studies have shown that BDNF expression is elevated in muscle fibers expressing slow type I myosin heavy chain (MyHC) (Mousavi and Jasmin, 2006; Gonzalez and Collins, 1997), which are known to have a higher number of associated satellite cells than fast MyHC IIB-expressing fibers (Charge and Rudnicki, 2004; Snow, 1983; Schmalbruch and Hellhammer, 1977). Taken together, these findings suggest an important role for BDNF in myogenic differentiation.

1.12 Statement of Hypothesis and Objectives

In order to elucidate the functional significance of BDNF in skeletal muscle, we have examined the effects of BDNF depletion in mouse models (described below) to test the hypothesis that **BDNF plays an important role in muscle development and regeneration**. The following objectives were formulated:

Objective 1: Generate a muscle-specific BDNF knockout mouse

In order to circumvent the problems of early postnatal mortality and breeding issues associated with the complete BDNF knockout mouse, we will use Cre/Lox technology to generate a mouse in which BDNF is specifically depleted in skeletal muscle cells that express the myogenic-determination gene, Myf5. These mice will allow close examination of postnatal muscle development and regeneration following injury.

Objective 2. Examine the role of BDNF in postnatal muscle development

In order to determine the functional significance of BDNF in skeletal muscle development, we will examine various histological and molecular characteristics of skeletal muscles derived from the muscle specific knockout mouse (BDNF^{MKO}) and complete BDNF knockout mice (BDNF^{-/-}) compared to their respective control littermates. In addition, single fibers and their associated satellite cells will be isolated and cultured. Assays of satellite cell activation, proliferation and differentiation will be performed to determine whether BDNF plays a functional role in regulating myogenic differentiation of primary myoblasts.

Objective 3. Examine the role of BDNF in injured/regenerating skeletal muscle.

Given the proposed role of BDNF in regulating myogenic differentiation of satellite cells, it will be important to examine the effects of BDNF depletion on postnatal myogenesis in vivo. For these experiments, muscle regenerative capacity will be examined at both the molecular and histological levels over a 1 week timecourse following cardiotoxin-induced injury.

CHAPTER 2

Materials & Methods

2. Materials and Methods

2.1 *BDNF*^{-/-} and Muscle-Specific *BDNF* Knockout Mice (*BDNF*^{MKO})

Heterozygous *BDNF*^{+/-} mice were purchased from Jackson laboratories (B6.129S4-*BDNF*^{tm1Jae/J}; Bar Harbor, ME). Homozygous *BDNF*^{-/-} mice exhibit severe neuronal defects caused by degeneration of sensory ganglia, and die within 2-3 weeks of birth. In our hands, *BDNF*^{+/-} mice were extremely aggressive towards littermates and pups, resulting in loss of the majority of litters within hours of birth. However, using selective breeding and environmental enrichment strategies, we were successful in obtaining a number of *BDNF*^{-/-} pups at P1. For this reason, all experiments involving *BDNF*^{-/-} animals were performed at P1.

For generation of muscle-specific *BDNF* knockout colonies, mice carrying the LoxP-targeted *BDNF* allele (*BDNF*^{ff}) (*BDNF*^{tm2Jae/J}; Jackson Labs, Bar Harbor, ME; Rios et al, 2001) were crossed with *Myf5*-Cre mice (kindly provided by M. Rudnicki, Sprott Center for Stem Cell Research, Ottawa; Tallquist et al, 2000) in which Cre recombinase gene is knocked into the endogenous *Myf* locus. Resulting *BDNF*^{ff/wt}; *Myf5*-Cre progeny were then backcrossed to *BDNF*^{ff} homozygotes in order to produce *BDNF*^{ff}; *Myf5*-Cre mice (*BDNF*^{MKO}), in which skeletal muscle-*BDNF* is specifically knocked out. To assess the efficiency of Cre-mediated *BDNF* excision, genomic DNA was PCR-genotyped using primers that flank the LoxP-targeted *BDNF* locus. *BDNF*^{wt/wt}; *Myf5*-Cre mice were used as controls (CTL) in all experiments. All animals were housed in the animal care facility at the University of Ottawa, and provided with unlimited access to food and water. Surgical procedures were approved by the University of Ottawa Animal Care and Use Committee.

2.2 Single Fiber Satellite Cell Isolation

Flexor digitorum brevis (FDB) muscles were removed from 5-6 week old BDNF^{MKO} and control mice and digested in a Petri dish containing type I collagenase (Sigma, St. Louis, MO) in DMEM (Gibco, Burlington, Ontario, Canada) for 1.5 hours. Single fibers and their associated satellite cells were isolated as previously described (Rosenblatt et al, 1995). Briefly, myofibers were dissociated by repeated titration in DMEM, and single intact fibers were cultured on Matrigel coated culture plates containing basal medium (10% horse serum, 1% antibiotic in DMEM). Within 2 days of plating, satellite cells displaying myogenic potential detached from single fibers and basal medium was replaced by growth medium containing 20% fetal bovine serum, 10% horse serum and 1% antibiotic in DMEM. Growth medium was changed daily for 5 days. Differentiation was induced following serum deprivation of cultures using 2% horse serum and 1% antibiotic in DMEM.

2.3 Cardiotoxin Injections

For regeneration experiments, 25ul of 10⁻⁵M cardiotoxin (Latoxan, Rosans, France) were injected into the tibialis anterior (TA) muscle of 5-6 week old BDNF^{MKO} and control mice to induce muscle degeneration and regeneration as previously described (Gramolini et al, 1999; Condrea 1974). One, two, five and seven days following injection, right TA muscles were excised and frozen in liquid nitrogen for RNA and/or protein extraction. For immunofluorescence experiments, left TA muscles were embedded in optimal cutting temperature (OCT) embedding medium (Fisher Scientific, Pittsburg, PA) and frozen in melting isopentane pre-cooled with liquid nitrogen. All muscles were stored at -80°C until use.

2.4 RNA Extraction and RT-PCR

RNA was extracted from muscle and brain using TRIzol reagent (Invitrogen) as recommended by the manufacturer. TRIzol extracted RNA was treated for 1 hour with DNase I (Invitrogen) to eliminate possible DNA contamination. Reverse transcription was carried out using an RT reaction containing 5mM MgCl₂, 1x PCR buffer, 1mM dNTP, 1U/ul RNase inhibitor, 5 U/ul MuV reverse transcriptase, and 2.5 uM random hexamers. Endpoint PCR amplification of endogenous transcripts was performed as previously described (Mousavi and Jasmin, 2006) using primers outlined in Table 4. Cycle numbers varied depending on the primers used, however all were optimized to be within linear range of amplification as previously described in detail (Mousavi et al, 2004). Controls consisted of RT mixture in which total RNA was replaced by diethylpyrocarbonate (DEPC)-treated water, and/or RT mixture in which reverse transcriptase was replaced with DEPC-treated water. PCR products were visualized on 1% agarose gel containing ethidium bromide, and labeling intensity of the PCR products was quantified using Kodak digital science 1D image analysis software.

Real-time quantitative RT-PCR was performed on a Stratagene MX3005p real-time PCR system using QuantiTect SYBR green PCR kit (Qiagen), as previously described (Miura et al, 2008). For these experiments, amplification of transcripts was performed in triplicate using the primers described in Table 2. Analysis was performed using Stratagene MxPro qPCR software (Agilent Technologies, Santa Clara, CA) The delta CT method was used to quantify expression levels based on normalization to 18S Ribosomal RNA (cardiotoxin experiments) and/or GAPDH.

Table 4. Oligonucleotides used in this study

QUANTITATIVE	RT-PCR	
BDNF	FWD	5'-CCATGACCTGAAAGGAAACAG-3'
	REV	5'-CACAAATTAAGCAGCATGCAA-3'
Pax7	FWD	5'-GACGACGAGGAAGGAGACAA-3'
	REV	5'-CGGGTTCTGATTCCACATCT-3'
embMyHC	FWD	5'-GCAGATTCAGAACTGGAGAC-3'
	REV	5'-CCTTAACACGCCGCTCATAC-3'
neoMyHC	FWD	5'-TCGCTGGCTTTGAGATCTTT-3'
	REV	5'-ACGAACATGTGGTGGTTGAA-3'
Myogenin	FWD	5'-ATCGCGCTCCTCCTGGTTGA-3'
	REV	5'-CTGGGGACCCCTGAGCATTG-3'
MyoD	FWD	5'-TGGCATGATGGATTACAGCG -3'
	REV	5'-CCACTATGCTGGACAGGCAGT-3'
P21	FWD	5'- CTCTTCTGCTGTGGGTCAGG -3'
	REV	5'- AAGTTTGGAGACTGGGAGAGG -3'
18S Ribosomal	FWD	5'-CGCCGCTAGAGGTGAAATC-3'
	REV	5'-CCAGTCGGCATCGTTTATGG-3'
GAPDH	FWD	5'-GGGTGTGAACCACGAGAAAT-3'
	REV	5'-CCTCCACAATGCCAAAGTT-3'

ENDPOINT	RT-PCR	
NT-3	FWD	3'-CGGATGCCATGGTTACTTACTTCT-5'
	REV	3'-AGCGTCTCTGTTGCCGTAGT-5'
NT-4/5	FWD	3'-AGAGTGAGGAGGTGGAGGTG-5'
	REV	3'-ACTCACTGCGTCGCATACTG-5'
NGF	FWD	3'-TGACTTTGGAGCTGGCCTTA-5'
	REV	3'-TCACTGCGGCCAGTATAGAA-5'
MyoD	FWD	3'-GCCCGCGCTCCAAGTCTCTGAT-5'
	REV	3'-CCTACGGTGGTGCGCCCTCTCGC-5'
Myogenin	FWD	3'-GGGCCCTGGAAGAAAAG-5'
	REV	3'-AGGAGGCGCTGTGGGAGT-5'
MSTN	FWD	3'-GACGATTATCACGCTACCACGGAAAC-5'
	REV	3'-CATCGCAGTCAAGCCCAAAGTCTCTC-5'
28S	FWD	3'-TTGTTGCCATGGTAATCCTGCTCAGTACG-5'
	REV	3'-TCTGACTTAGAGGCGTTCAGTCATAATCCC-5'

2.5 Western Blotting

Total protein was extracted from muscle and brain samples using RIPA extraction buffer (150mM NaCl, 1% IPGAL, 0.5% sodium deoxycholate, 0.1% SDS, 50mM Tris (pH 8.0)) supplemented with protease inhibitors (Complete; Roche). Protein concentration was determined using Bradford assay (BioRad, Hercules, CA). Samples (30ug) were run on 12% SDS-page gels, and transferred to polyvinylidene difluoride membrane. Western blot analysis was performed as previously described (Mousavi and Jasmin, 2006). Briefly, membranes were blocked with 5% non-fat milk in Tris-buffered saline and Triton X-100, before incubation with either α -Pax7 (Developmental Studies Hybridoma Bank [DSHB]) or α -GAPDH primary antibodies (Advanced Immunochemical, Long Beach, CA) antibodies. Secondary detection was performed using HRP-conjugated secondary antibodies (Chemicon International, Temecula, CA). Antibody complexes were detected using western lighting chemiluminescent reagent (Perkin Elmer, Waltham, MA) and exposed to Kodak BioMAX MR film (Eastman Kodak, Rochester, NY). Quantification of band density was performed using Image J image analysis software.

2.6 ELISA

BDNF protein was quantified from muscle and brain samples using the Emax ImmunoAssay system (Promega, Madison, WI) as previously described (Mousavi and Jasmin, 2006). Briefly, samples were homogenized in lysis buffer (2:1 vol/wt) containing 137mM NaCl, 20mM Tris-HCl (pH 8.0), 1% NP-40, 10% glycerol, 1mM PMSF, 0.5 mM sodium vanadate, and one protease inhibitor tablet (Roche, Indianapolis, IN). Samples were diluted with 4 volumes of Dulbecco's phosphate-buffered saline, sonicated, and centrifuged at 14000g for 20 minutes.

Supernatant was collected and quantified using bicinchonic acid (BCA) method (Pierce, Rockford, IL). ELISA detection was performed following the instructions of the manufacturer. Briefly, 96-well plates were coated with 1 μ g/ml monoclonal BDNF antibody. Plates were then blocked for 1 hour before incubation with protein samples and standards for 2 hours at room temperature. Following sample incubation, plates were washed, and incubated for another 2 hours with 0.5 μ g/ml of polyclonal BDNF antibody. Plates were washed thoroughly and incubated with an anti-IgY horseradish peroxidase-conjugated antibody (1:200) for 1 hour. Finally, reactions were developed using TMB One solution for 15 minutes, and sample absorbance was measured at 450nm with an ELISA plate reader (SpectraMax M2).

2.7 Muscle Histology

For all histological procedures, muscles were removed from animals, immediately frozen in OCT embedding medium (Fisher Scientific, Pittsburgh, PA) and stored at -80°C until use. Muscles were cross-sectioned at 10 μ m thickness and placed on positively charged slides for histological examination. Bright field and fluorescent images were digitally acquired (Sony) using a Zeiss Axioscope 2 microscope. Analysis was performed using Northern Eclipse Imaging Software, and further image processing software was used to enhance color and clarity for publication (Photoshop CS, Adobe). All histological assessments were performed in a blinded fashion such that the identity of the samples remained unknown until data analysis was complete.

For visualization of overall muscle structure, sections were stained with hematoxylin and eosin. The average number of newly regenerated myofibers was quantified as the average

number of centrally nucleated fibers counted across 5 separate fields of view for at least three sections of each muscle.

2.8 Immunofluorescence

Immunofluorescent labeling was performed using primary antibodies against BDNF (Promega, Madison, WI), CD11b (Abcam, Cambridge, MA) and various myosin heavy chain proteins (see below; Developmental Studies Hybridoma Bank, Iowa City, IA). Muscle sections were fixed with 2% PFA for 10 minutes at room temperature and blocked (1% BSA, 0.1% Triton-X in PBS) for an additional hour. Primary antibody incubation was performed at 37°C for 1 hour, followed by incubation with fluorescently conjugated secondary antibodies for 1 hour at room temperature.

Immunofluorescent fiber type analysis was performed using primary antibodies for myosin heavy chains (MyHC) I (A4-840; Developmental Studies Hybridoma Bank [DSHB], University of Iowa), IIA (SC-71) and IIB (BF-F3; German Collection of Microorganisms and Cell Cultures, Germany) and MyHC IIX (kindly provided by J.M. Renaud, University of Ottawa, Ontario). In addition, sections were co-stained with an antibody directed against laminin (Sigma, St. Louis, Missouri) an extracellular matrix protein that allows delineation of fiber boundaries. Secondary detection was performed using FITC-conjugated goat anti-mouse antibodies (Chemicon) to detect α -MyHC, while TRITC-conjugated anti-rabbit antibody was used to detect α -laminin staining. Analysis of fiber-type proportion was performed as previously described in detail (Mousavi et al, 2002).

For culture experiments, cells were fixed with 90% cold methanol and blocked for 1 hour at room temperature. For immunofluorescent detection, samples were incubated in primary antibodies overnight at 4°C. The next day sections were washed with PBS and incubated in appropriate secondary antibodies for 1 hour. Slides were mounted with Vectashield containing 4',6-diamidino-2-phenylindole (DAPI) stain to visualize nuclei (Vector Labs, Burlington, ON).

2.9 BrdU Incorporation Experiments

Bromodeoxyuridine (BrdU) labeling was performed using a BrdU labeling and detection kit, as described in the manufacturer's protocol (Roche, Indianapolis, IN). Following 5 days in growth promoting medium, cells were incubated with either growth (20% FBS) or differentiation (2% HS) promoting medium for 24 hours. BrdU labeling reagent (1ul/ml) was added 30 minutes prior to fixation with 70% ethanol in 50mM glycine (pH 2.0) for 20 minutes at -20°C. Cells were then washed with PBS and incubated with anti-BrdU monoclonal antibody for 30 minutes at 37°C. Secondary detection was performed using an anti-mouse-fluorescein for 30 minutes at 37°C. Slides were mounted with Vectashield (Vector Labs, Burlington, ON).

2.10 Statistical Analysis

Two-tailed Student's t tests were used to determine the significance of the difference between the two groups. All statistical analysis was performed using Microsoft Excel, with the levels of significance was set at $p < 0.05$.

CHAPTER 3

Results

Publication Notice and Reproduction Authorization

A large component of the data presented in Chapter 3 has been accepted for publication in *Molecular Biology of the Cell* (MBC) in the following article:

Clow C. and Jasmin B.J. (2010) Skeletal Muscle-Derived BDNF Regulates Satellite Cell Differentiation and Muscle Regeneration. *Mol. Biol. Cell.* In Press

3. Results

3.1 Generation of the Muscle-Specific BDNF Knockout Mouse

In order to examine the role of skeletal muscle-derived BDNF in postnatal muscle development, maintenance and regeneration, we generated a mouse in which BDNF is specifically depleted from skeletal muscle cells. To this end, mice homozygous for the LoxP-targeted BDNF allele ($BDNF^{fl/fl}$) were crossed with Myf5-Cre knockin mice, in which Cre recombinase expression is driven by the muscle-specific Myf5 promoter (Tallquist, 2000; Huh et al, 2004). Genomic DNA was amplified using primers to detect the Cre transgene, and primers that flank the LoxP-targeted BDNF locus. Heterozygous $BDNF^{fl/wt}$; Myf5-Cre progeny were then backcrossed to $BDNF^{fl/fl}$ mice to generate $BDNF^{fl/fl}$; Myf5-Cre mice ($BDNF^{MCKO}$), in which muscle-BDNF is specifically depleted (Fig. 5).

$BDNF^{MCKO}$ mice were viable and fertile, and were produced at expected Mendelian ratios. Cre-mediated excision of the BDNF gene was assessed at postnatal day 7 (P7) and in adult hindlimb muscles using quantitative RT-PCR (qRT-PCR) with primers to exon 5 of the BDNF gene. At P7, BDNF transcript levels were decreased by approximately 50% in whole hindlimb preparations of $BDNF^{MCKO}$ mice compared to control littermates (Fig. 6A, $p < 0.01$). Greater decreases in BDNF transcript (~60%) were detected in isolated extensor digitorum longus (EDL), gastrocnemius (GAS), tibialis anterior (TA), soleus (SOL) and diaphragm (DIA) muscles of adult (5-8 week) $BDNF^{MCKO}$ mice (Fig. 6A, $p < 0.01$). Specificity of the BDNF knockout to muscle cells was confirmed using qRT-PCR and ELISA on brain samples isolated from $BDNF^{MCKO}$ and control littermates. Importantly, BDNF expression was similar at both the transcript and protein level between $BDNF^{MCKO}$ and control littermates (Fig. 6A and B). In

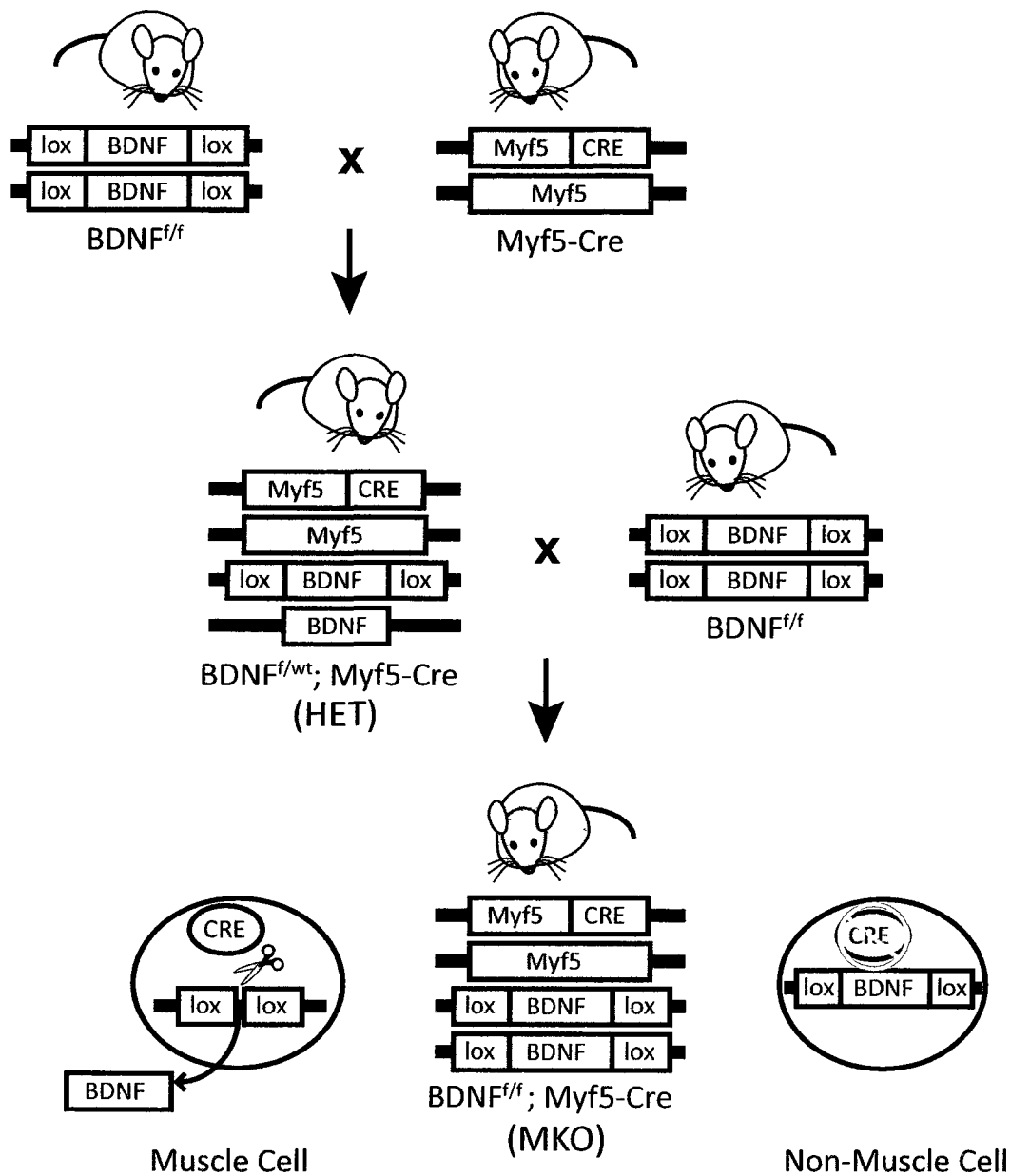


Figure 5. Generation of the muscle-specific BDNF knockout mouse. Mice homozygous for the LoxP-targeted BDNF allele ($BDNF^{f/f}$) were crossed with Myf5-Cre knockin mice. Heterozygous (HET) $BDNF^{f/wt}; Myf5-Cre$ progeny were then backcrossed to $BDNF^{f/f}$ homozygotes in order to generate $BDNF^{f/f}; Myf5-Cre$ mice (MKO) in which muscle-BDNF is specifically depleted. $BDNF^{wt/wt}; Myf5-Cre^+$ littermates, in which the Cre transgene is present but no LoxP target sites exist, were used as controls.

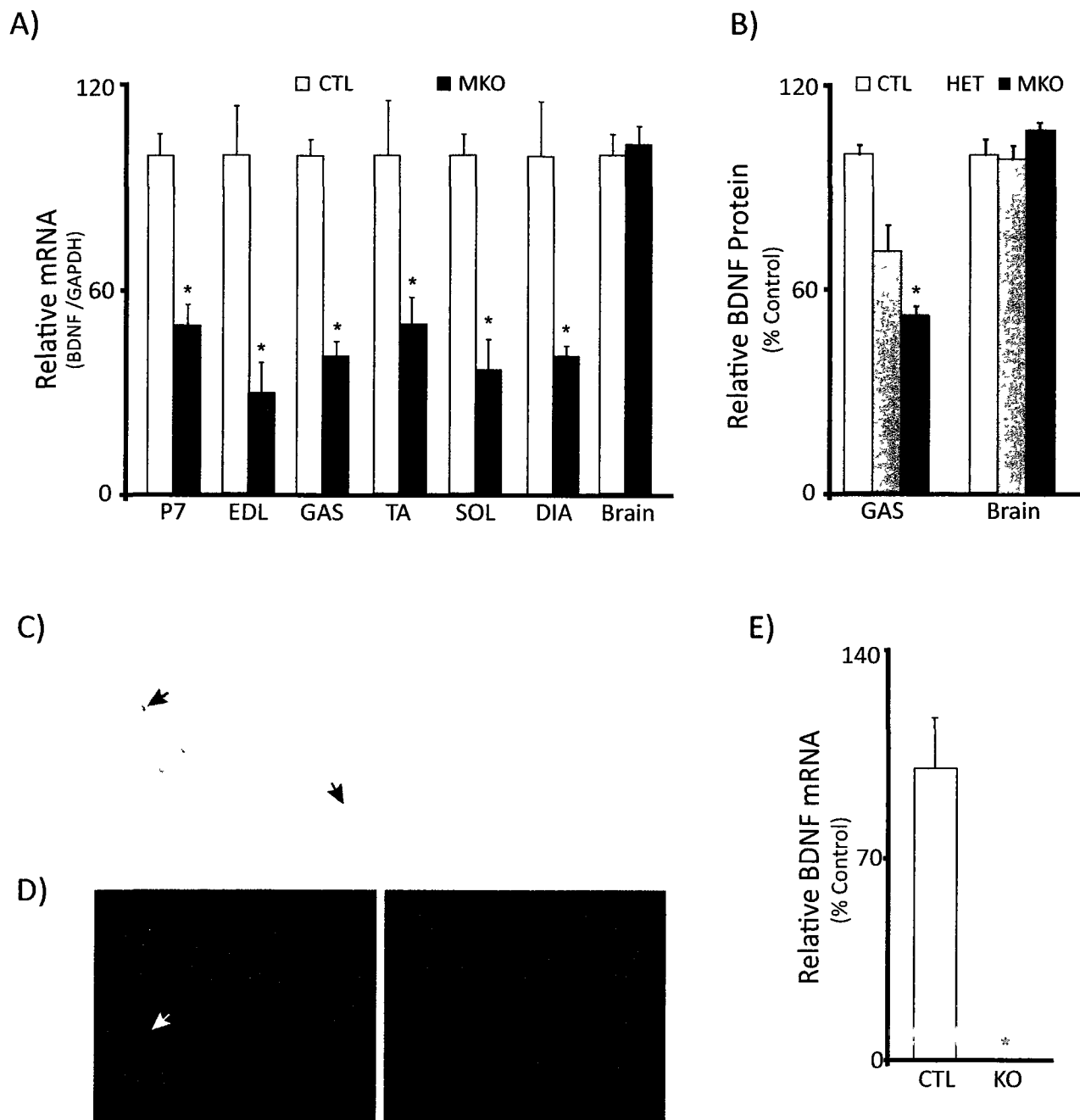


Figure 6. BDNF depletion from hindlimb muscles of the BDNF^{MKO} Mouse. A) Quantification of the relative levels of BDNF mRNA detected by quantitative RT-PCR in total hindlimb musculature at postnatal day 7 (P7) and in adult (5-8 week old) extensor digitorum longus (EDL), gastrocnemius (GAS), tibialis anterior (TA), soleus (SOL), and diaphragm (DIA) muscles of BDNF^{MKO} (MKO) animals compared to control littermates (n=6, *p<0.01) B) Relative levels of BDNF protein per milligram wet tissue mass in adult gastrocnemius (GAS) muscle and brain preparations from CTL, heterozygous (HET), and MKO mice (n=4, *p<0.05). C) Representative immunoperoxidase staining of BDNF protein in TA cross sections taken from adult CTL and MKO mice. Arrows indicate BDNF labelling. D) Immunofluorescent labelling of BDNF (Green) in CTL and MKO TA cross sections. Images are counterstaining with 4',6-diamidino-2-phenylindole (DAPI) in blue (n=3). E) Relative levels of BDNF mRNA detected in hindlimb musculature of BDNF^{-/-} (KO) compared to control littermates (CTL) at postnatal day 1 (n=3, *p<0.001). Error bars represent standard error of the mean (SEM).

skeletal muscle, ELISAs detected a 25% decrease in BDNF protein in gastrocnemius muscles isolated from heterozygous $BDNF^{fl/wt}$; Myf5-Cre animals, and an approximate 50% decrease in $BDNF^{fl/fl}$; Myf5-Cre ($BDNF^{MKO}$) muscles compared to control littermates (Fig. 6B, $p < 0.05$). BDNF protein was below the level of detection in $BDNF^{MKO}$ cross-sections of tibialis anterior muscles using immunofluorescent detection methods (Fig. 6C and D). However, BDNF-staining was readily detected in muscles isolated from control littermates, with $BDNF^+$ cells appearing as peripheral nuclei within the satellite position of muscle fibers (Fig. 6C and D).

Finally, we examined hindlimb preparations from complete BDNF knockout mice ($BDNF^{-/-}$) in order to demonstrate the relative abundance of detectable BDNF in $BDNF^{MKO}$ muscle compared to $BDNF^{-/-}$ animals. In our hands, $BDNF^{-/+}$ mice were extremely aggressive towards littermates and pups, resulting in loss of the vast majority of litters within a few hours after birth. However, using selective breeding and environmental enrichment, we were successful in obtaining $BDNF^{-/-}$ pups at postnatal day 1 (P1). As expected, we were unable to detect BDNF transcript in $BDNF^{-/-}$ animals compared to ~40-50% remaining BDNF in $BDNF^{MKO}$ muscles (Fig. 6E, $p < 0.0001$). Thus, it is important to note that compared to $BDNF^{-/-}$ muscle, where BDNF is completely abolished, detectable levels of BDNF remain in $BDNF^{MKO}$ muscles, which may allow for some BDNF signalling to persist (see below).

3.2 BDNF is Depleted from Satellite Cells of the $BDNF^{MKO}$ Mouse

Based on the observation that BDNF levels were only reduced by 50-60% in $BDNF^{MKO}$ muscles compared to 100% knockout in $BDNF^{-/-}$ animals, it became important to determine the source of remaining BDNF expression in the muscle-specific knockout model. Considering that

the muscle compartment is comprised of a number of cell types (Schwann, endothelial, neuronal, immune), and because a number of these cell-types have been shown to express and secrete BDNF, we hypothesized that the remaining BDNF detected in BDNF^{MKO} mice was derived from resident non-muscle cells (Lomen-Hoerth and Shooter, 1995; Vega et al, 2003; Kermani and Hempstead, 2007; reviewed; Gordon, 2009). Unfortunately, co-immunolabelling studies were not possible, as BDNF was below the level of detection using standard immunohistochemical techniques (Fig. 6C and D). Instead, we isolated and cultured single myofiber explants, in order to ensure that BDNF expression was indeed eliminated from skeletal muscle cells (Rosenblatt et al, 1995). These preparations allowed for the examination of a relatively pure sample of muscle fibers and their associated satellite cells in the absence of contaminating non-muscle cells. As expected, we found greater reductions than whole muscle preparations (85%, $p < 0.01$ compared to 50-60%, $p < 0.01$) in BDNF expression in cultures derived from BDNF^{MKO} mice, whereas BDNF was highly expressed in proliferating control cultures (Fig. 7). The remaining (15%) BDNF expression detected in BDNF^{MKO} cultures may be due to low levels of contaminating non-muscle cells (ie. fibroblasts, neurons, immune cells, Schwann cells). Alternatively, the remaining BDNF may be derived from a recently described population of satellite cells that never express Myf5, which could continue to express BDNF in the BDNF^{MKO} mouse (Kuang et al, 2007). This Myf5-naïve population has been shown to comprise approximately $13 \pm 4\%$ of the total Pax7⁺ satellite cell population, which correlates well with the remaining 15% control BDNF levels detected in BDNF^{MKO} cultures. Together, these findings suggest that approximately 35% of BDNF detected in whole muscle preparations is derived from resident non-muscle cells, while the remaining 65% is normally expressed by satellite cells, 15% of which do not express Myf5.

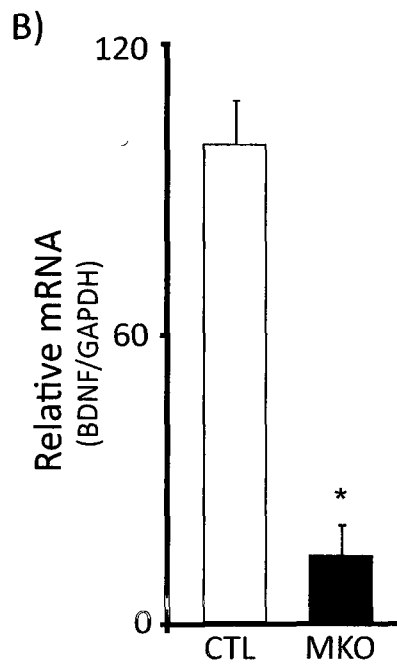
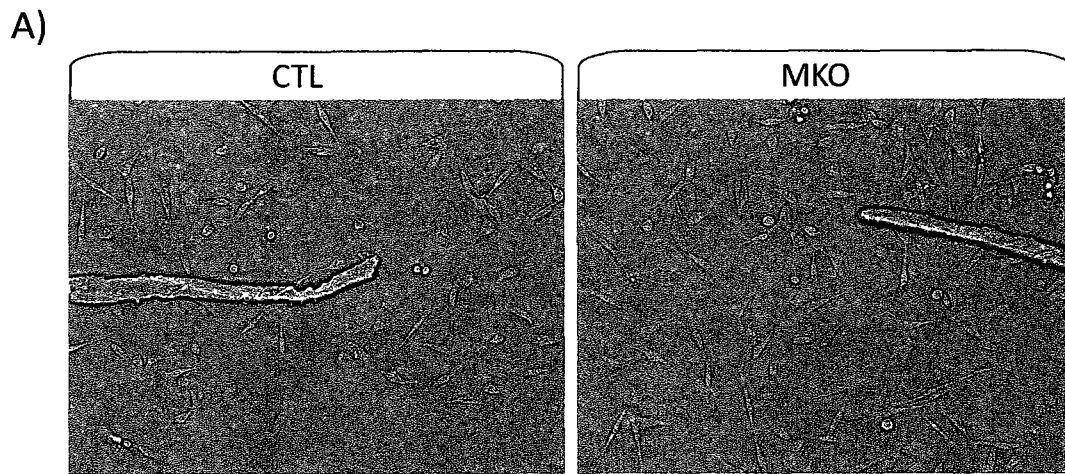


Figure 7. BDNF depletion from single fiber myoblast preparations. A) Representative phase contrast images of control (CTL) and BDNF^{MKO}(MKO) -derived single fibers and associated myoblasts following 5 days growth (n=3). B) Relative levels of BDNF mRNA detected in CTL and MKO single fiber myoblast preparations (n=6, p<0.01).

3.3 Expression of Neurotrophin Family Members and their Receptors in the BDNF^{MKO} Mouse

The neurotrophins are structurally and functionally related, and although they bind predominantly to their respective Trk receptors, considerable cross-talk between certain neurotrophins and receptors has been reported (reviewed, McDonald and Chao, 1995; Butte, 2001). In particular, changes in stoichiometric levels of certain neurotrophins or receptors can result in promiscuous activation of downstream signalling cascades (Soppet et al, 1991; Squinto et al, 1991; Stohmaier et al, 1996; Arevalo et al, 2006). As such, expression levels of other neurotrophins and/or their receptors could be altered to compensate for the loss of BDNF in the muscle-specific knockout model to allow binding and activation of signalling cascades that normally rely on BDNF for transduction. For this reason, we examined the expression profile of neurotrophin family members (BDNF, NT-3, NT-4/5, NGF) and their receptors (TrkA, TrkB, TrkC and p75^{NTR}) to determine whether expression levels were adjusted in the BDNF^{MKO} mouse. Importantly, we did not detect any significant changes in the expression level of neurotrophin family members or neurotrophin receptors in adult BDNF^{MKO} hindlimb muscles compared to controls (Fig. 8, $p > 0.05$). This suggests that the expression of other neurotrophin family members, particularly NT-4/5, which binds the same receptors (TrkB and p75^{NTR}) as BDNF, is not altered in order to compensate for the loss of BDNF in the BDNF^{MKO} mouse.

3.4 Hindlimb Muscle Morphology in the Muscle-Specific BDNF Knockout Mouse

The muscle-specific BDNF knockout mouse showed no overt phenotypic abnormalities, and was similar in weight and size to control littermates from birth to 1 year old. Histological examination of hematoxylin and eosin stained cross-sections of SOL and EDL muscles from P7

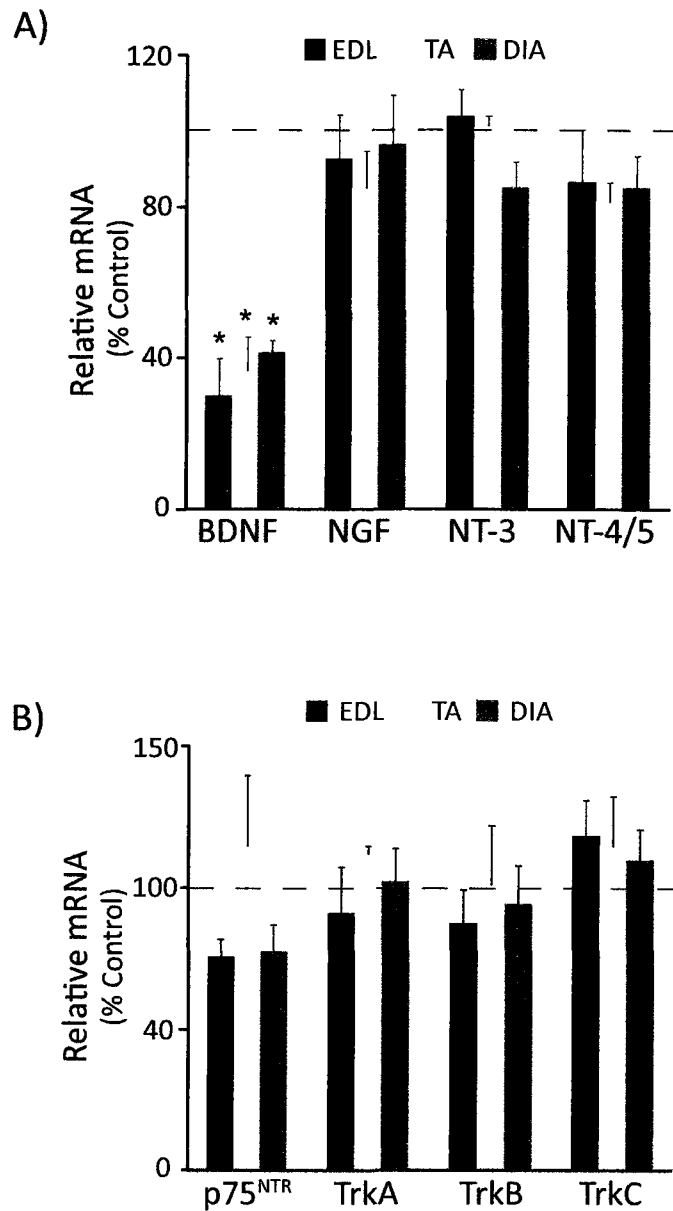


Figure 8. Expression levels of neurotrophin family members and their receptors are not altered in the BDNF^{MKO} mouse. A) Relative levels of BDNF, NGF, NT-3 and NT-4/5 transcript in extensor digitorum longus (EDL), tibialis anterior (TA), and diaphragm (DIA) muscles in adult BDNF^{MKO} compared to control (- -) levels. B) Relative levels of neurotrophin receptors; p75^{NTR}, TrkA, TrkB and TrkC in the BDNF^{MKO} mouse. All values are relative to control mRNA levels standardized to 28S ribosomal RNA (n=6) Error bars represent SEM.

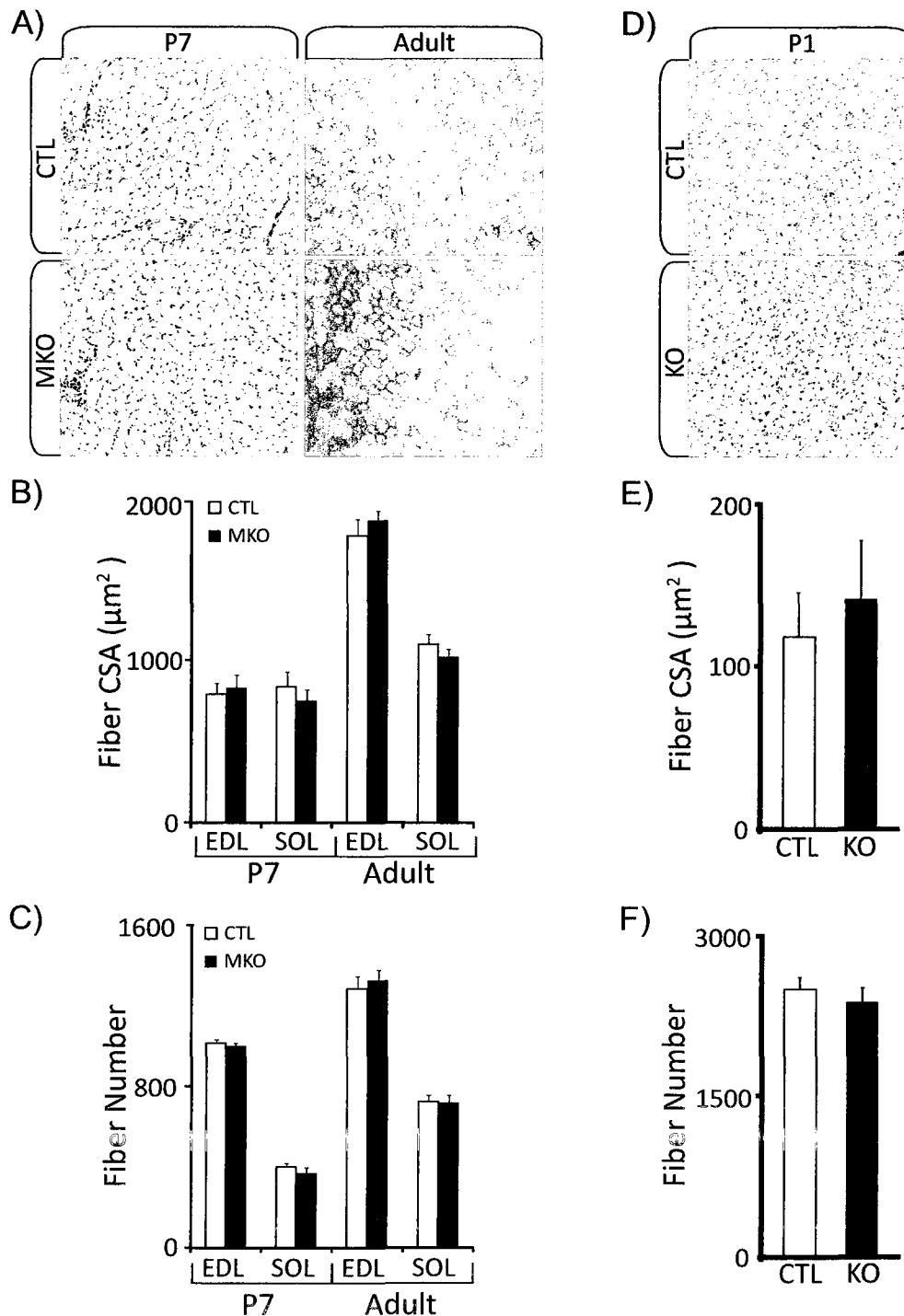


Figure 9. Overall muscle histology is not affected in the absence of muscle-BDNF. A) Representative hematoxylin and eosin stained extensor digitorum longus (EDL) cross-sections from control (CTL) and $BDNF^{MKO}$ (MKO) hindlimb sections in at postnatal day 7 (P7, left panels) and adult (right panels). B) Quantification of the average cross-sectional area (CSA) and C) fiber number from P7 and adult EDL and soleus (SOL) muscles ($n=6$). D) Representative tibialis anterior (TA) cross-sections of $BDNF^{-/-}$ (KO) and control TA cross-sections at postnatal day 1 (P1). E) Quantification of CSA and F) fiber number from CTL and KO cross-sections at P1 ($n=3$). Error bars represent SEM.

and adult BDNF^{MKO} mice revealed no significant change in total myofiber size or number when compared to control littermates (Fig. 9A-C). Similar results were obtained upon examination of hindlimb muscles of BDNF^{-/-} full knockout mice at postnatal day 1 (Fig. 9D-F). Together, these findings suggest that overall muscle fiber number and size are not adversely affected in the absence of BDNF.

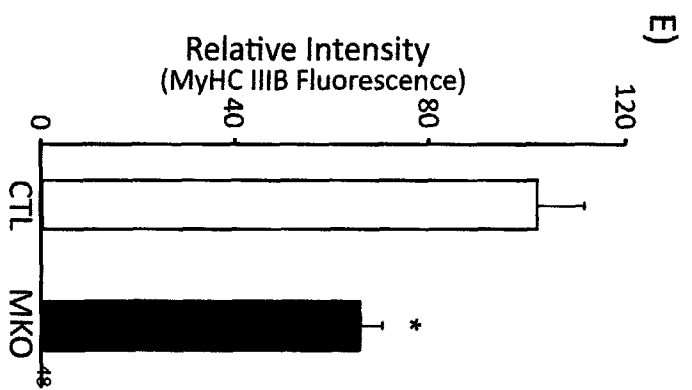
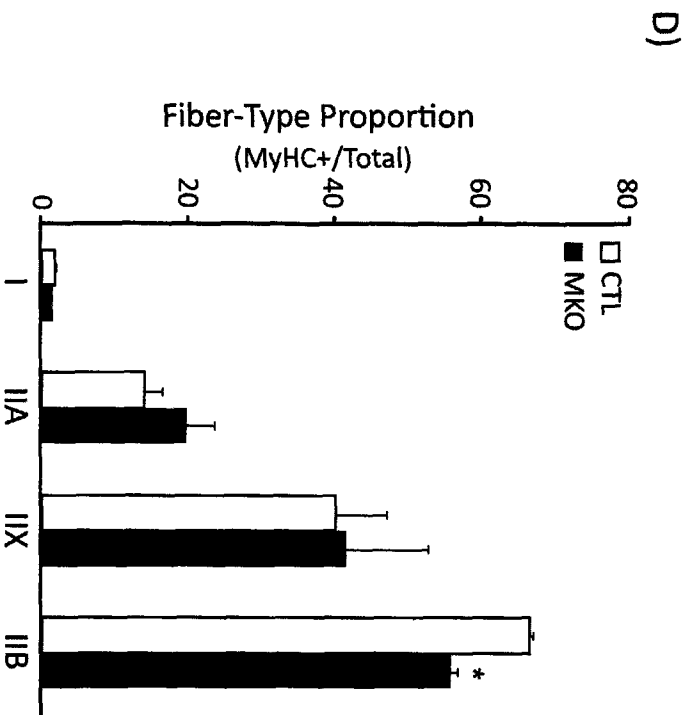
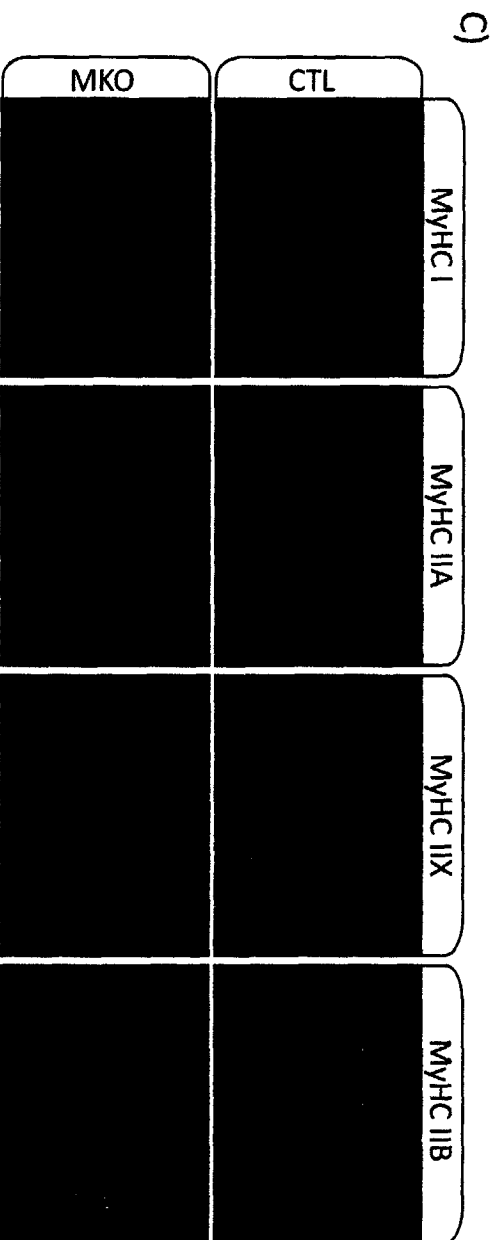
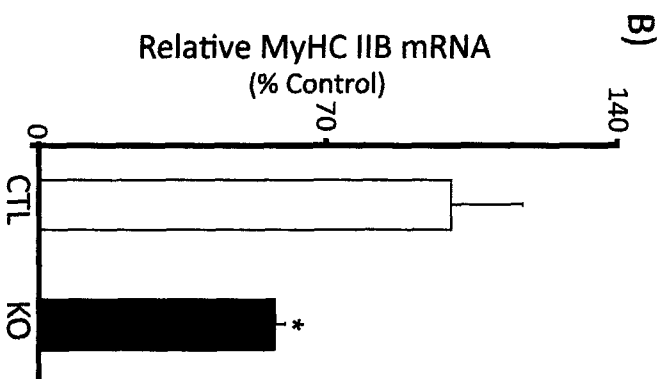
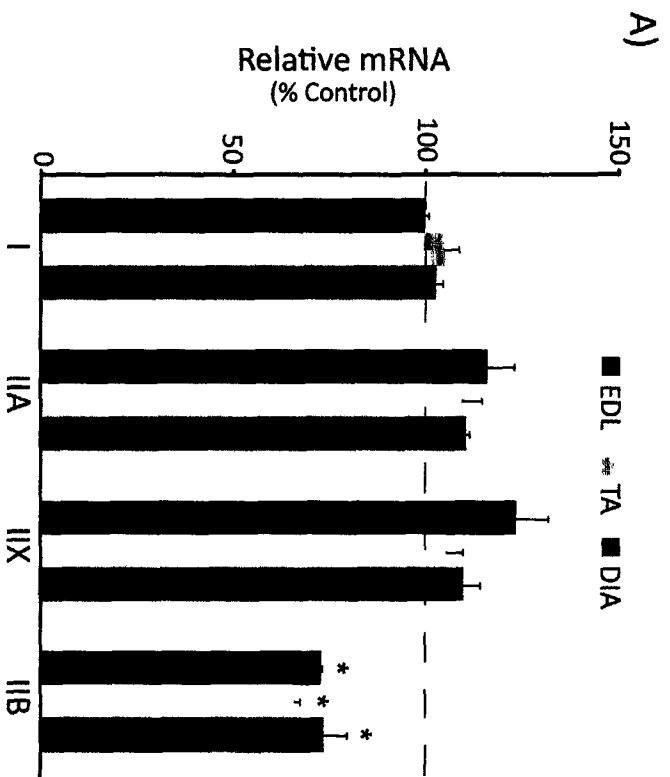
3.8 Changes in Myosin Heavy Chain Expression in BDNF^{MKO} and BDNF^{-/-} Mice

The contractile properties of skeletal muscle depend heavily on the composition and expression level of myosin heavy chain (MyHC) protein isoforms. Changes in MyHC expression profile can confer changes in the functional properties of muscle, and conversely, changes in functional demand can result in changes in MyHC composition (reviewed; Pette and Staron, 2000). Previous studies have implicated the neurotrophic factor NT-4/5 in fiber type transformation, specifically in promoting expression of slow type I MyHC (Carrasco and English, 2003). Because BDNF and NT-4/5 share common receptor binding properties, we next examined the MyHC expression profile of adult BDNF^{MKO} mice in order to determine whether BDNF plays a similar role in fiber type specification. Using quantitative RT-PCR we found that in the absence of muscle BDNF, transcript levels of the fast-twitch MyHC IIB isoform were reduced by approximately 25% compared to control littermates (Fig. 10A, $p < 0.01$). Conversely, other fast-twitch MyHC isoforms (MyHC IIX and MyHC IIA) showed a subtle, but statistically insignificant increase at the transcript level (Fig. 10A). A similar decrease in MyHC IIB expression was detected in BDNF^{-/-} animals at P1, where initial patterning of MyHC expression is ongoing (Fig. 10B, $p < 0.01$).

To determine whether changes in MyHC expression were reflected at the protein level, we performed immunofluorescent fiber type analysis using antibodies directed against MyHC I, IIA, IIX and IIB on cross-sections of EDL muscles isolated from BDNF^{MKO} and control littermates. Fiber type analysis revealed that the proportion of MyHC IIB-positive fibers was significantly reduced in the absence of muscle-BDNF (Fig. 10C and D, $p < 0.05$). Similarly, total fluorescence intensity of MyHC IIB-stained sections, which is reflective of the total amount of detectable protein, was decreased by more than 25% in BDNF^{MKO} sections (Fig. 10E, $p < 0.05$). Together, these findings suggest that BDNF plays an important role in determining fiber type composition of skeletal muscle.

Physiological properties of skeletal muscle depend heavily on electrochemical stimulation by innervating motor neurons (reviewed, Talmadge, 2000). We therefore hypothesized that the detected decrease in MyHC IIB expression in BDNF-depleted skeletal muscles may be an indirect effect of abnormal innervation of type IIB fibers. In order to examine properties of MyHC IIB fibers, we performed immunofluorescent staining for various pre and post-synaptic neuromuscular junction (NMJ) proteins on fiber-bundles preparations from the predominantly fast-glycolytic EDL muscle. Isolated fiber bundles were comprised of EDL fibers with intact innervating motor neurons and peri-synaptic Schwann cells. Pre-synaptic staining with α -neurofilament and α -synapsin a/b appeared normal on BDNF^{MKO} fibers (Fig. 11A and B). However, post-synaptic staining of acetylcholine receptor (AChR) clusters using α -bungarotoxin (BTX) revealed a number of morphological abnormalities (Fig. 11C). These results suggest that muscle-derived BDNF is required for normal formation and/or maintenance of the NMJ, particularly on fibers expressing MyHC IIB.

Figure 10: Decreased expression of fast MyHC IIB in BDNF-depleted skeletal muscle. A) Relative mRNA levels of MyHC I, IIA, IIX, and IIB in EDL, TA and DIA muscles from adult BDNF^{MKO} mice compared to 100% control (----) levels (n=5, *p<0.01). B) Quantification of the relative levels of MyHC IIB transcript in P1 hindlimb muscles isolated from BDNF^{-/-} and control littermates (n=3, p<0.05). C) EDL muscles were removed from adult BDNF^{ff}; Myf5-Cre and control littermates, frozen in OCT and cryosectioned at 10um. Immunofluorescent staining of CTL (top) and KO (bottom) EDL cross sections using anti- MyHC type I, IIA, IIX and IIB respectively; detected using FITC conjugated secondary antibodies (Green). Sections were co-stained with anti-laminin and detected using a TRITC conjugated secondary antibody (Red) in order to label the extracellular matrix, allowing delineation of muscle fiber boundaries. D) Quantification of MyHC fiber type proportions in fluorescently labelled cross sections. Values represent the percentage of positive fibers relative to total fiber number. E) Relative fluorescence intensity calculated for cross sections stained with antibodies to detect MyHC IIB protein. (n=3 for histological quantification, *p<0.05).



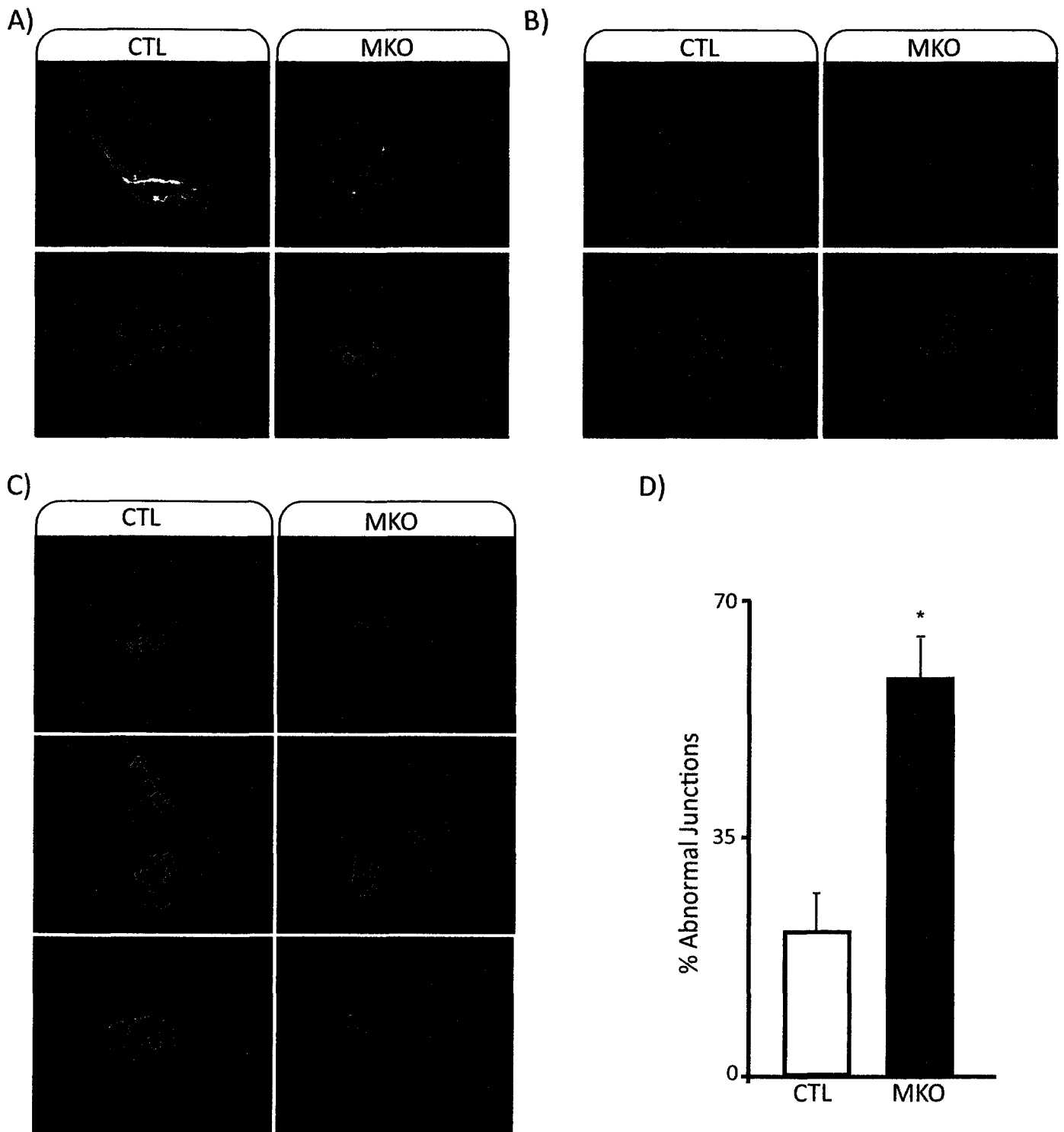


Figure 11. Neuromuscular junction abnormalities in the absence of muscle-BDNF. A) Immunofluorescent labeling of post-synaptic acetylcholine receptor using α -bungarotoxin (Green) and pre-synaptic neurofilament (red) on single fibers isolated from EDL muscles of BDNF^{MKO} (MKO) and control (CTL) littermates. B) Postsynaptic utrophin staining (red) colocalizes with BTX (green) in both MKO and CTL sections. C) Representative images of BTX-stained neuromuscular junctions. Images are counterstained with DAPI to reveal postsynaptic nuclei. D) Quantification of the number of abnormal junctions in MKO and CTL sections (n=6, p<0.05). Error bars represent SEM.

3.6 Expression of Muscle Regulatory Genes in the Muscle-Specific BDNF Knockout Mouse

To determine whether depletion of muscle-derived BDNF resulted in changes in the expression of genes controlling muscle growth and differentiation, we examined transcript levels of the myogenic regulatory factors (MRFs) in EDL, TA, and DIA muscles of adult BDNF^{MKO} and control mice. We observed no significant changes in transcript levels for positive regulators of myogenesis (MyoD and myogenin) in healthy adult skeletal muscle (Fig. 12). Likewise, transcript levels of myostatin, a negative regulator of muscle growth showed no significant change compared to control littermates (Fig. 12).

In contrast, the transcript and protein levels for Pax7, a well-known satellite cell marker, were consistently decreased in adult BDNF^{MKO} muscle compared to controls. Although there were no changes in Pax7 expression in neonatal (P7) BDNF^{MKO} muscles, adult transcript levels were decreased by 15% (Fig. 13A, $p < 0.05$), while Pax7 protein levels showed a 30% reduction compared to controls (Fig. 13B). Similar changes in Pax7 expression were observed in satellite cell-derived myoblast cultures following single fiber isolation (Fig. 13A and Fig. 14A, $p < 0.01$). For comparative purposes, we also examined Pax7 transcript levels in neonatal (P1) BDNF^{-/-} hindlimb muscle. By P1, Pax7 transcript levels were decreased by 30%, suggesting a more pronounced deficit in Pax7 expression when BDNF is completely abolished (Fig. 13C).

3.7 Muscle-BDNF is Required for Normal Satellite Cell Function

The basic-helix-loop-helix transcription factor Pax7 plays an important role in regulating satellite cell function. Pax7 germline mutants fail to produce muscle satellite cells, severely compromising postnatal growth and regenerative potential of these mice (Seale et al, 2000;

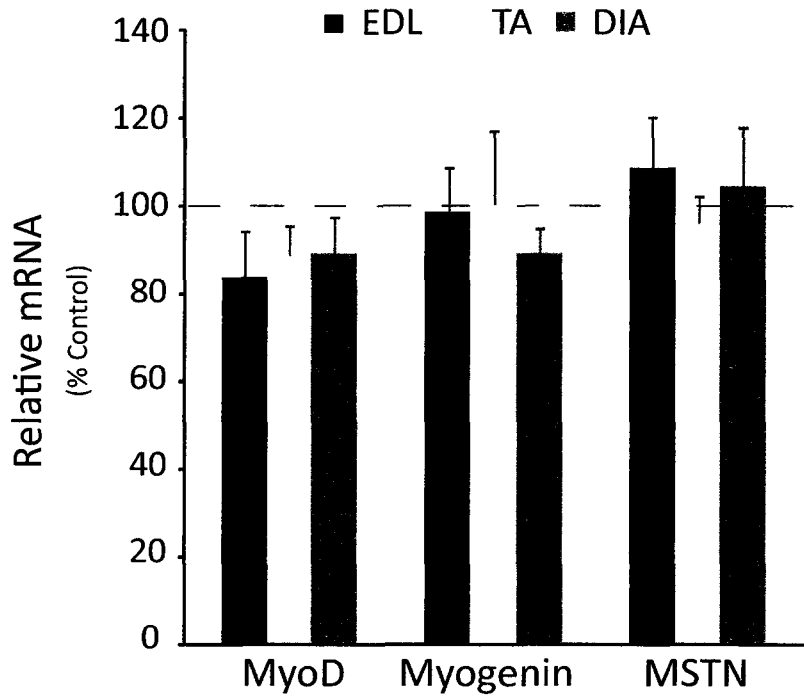


Figure 12. Expression levels of genes involved in regulation of myogenic differentiation are not changed in the $BDNF^{MKO}$ mouse. Relative levels of MyoD, Myogenin, and Myostatin (MSTN) transcript in EDL, TA, DIA muscles of adult $BDNF^{MKO}$ mice. All values are relative to control mRNA levels (---) standardized to 28S ribosomal RNA (n=6) Error bars represent SEM.

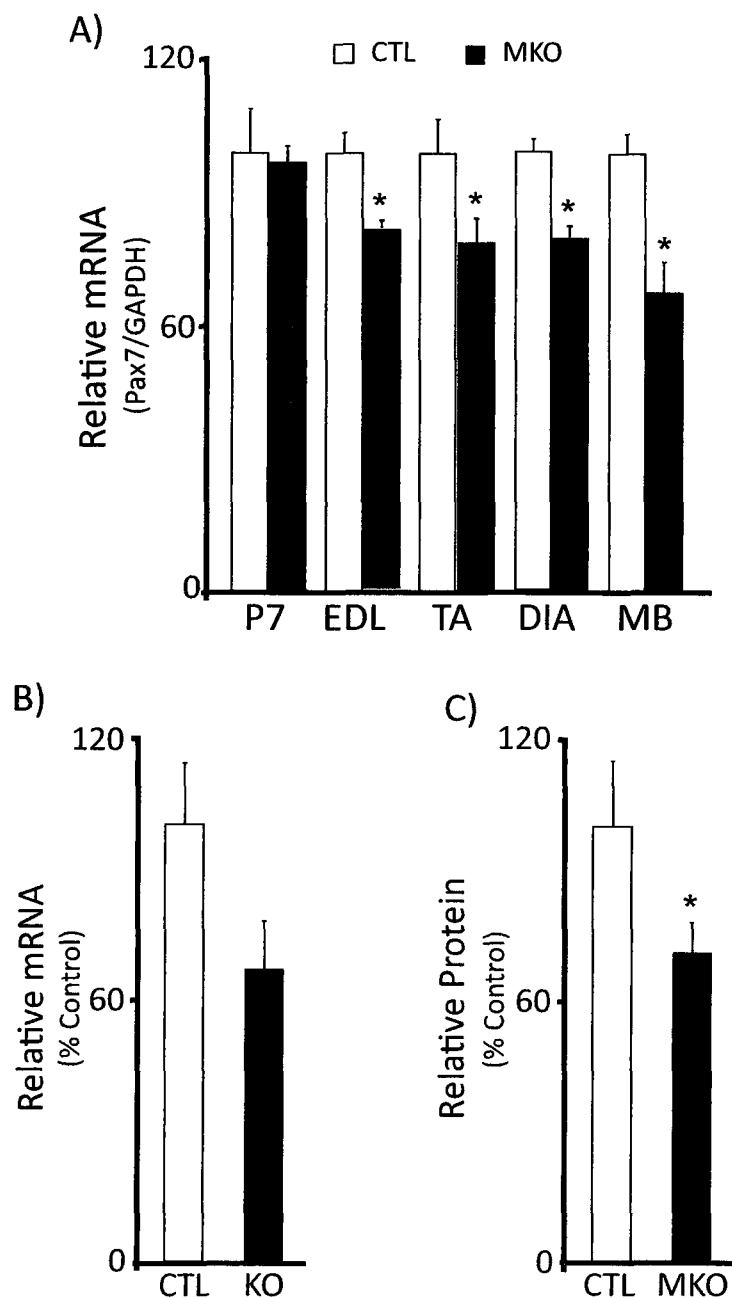
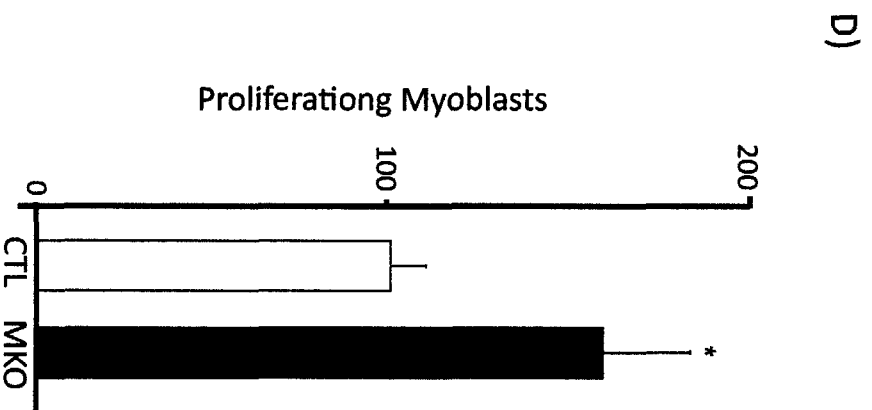
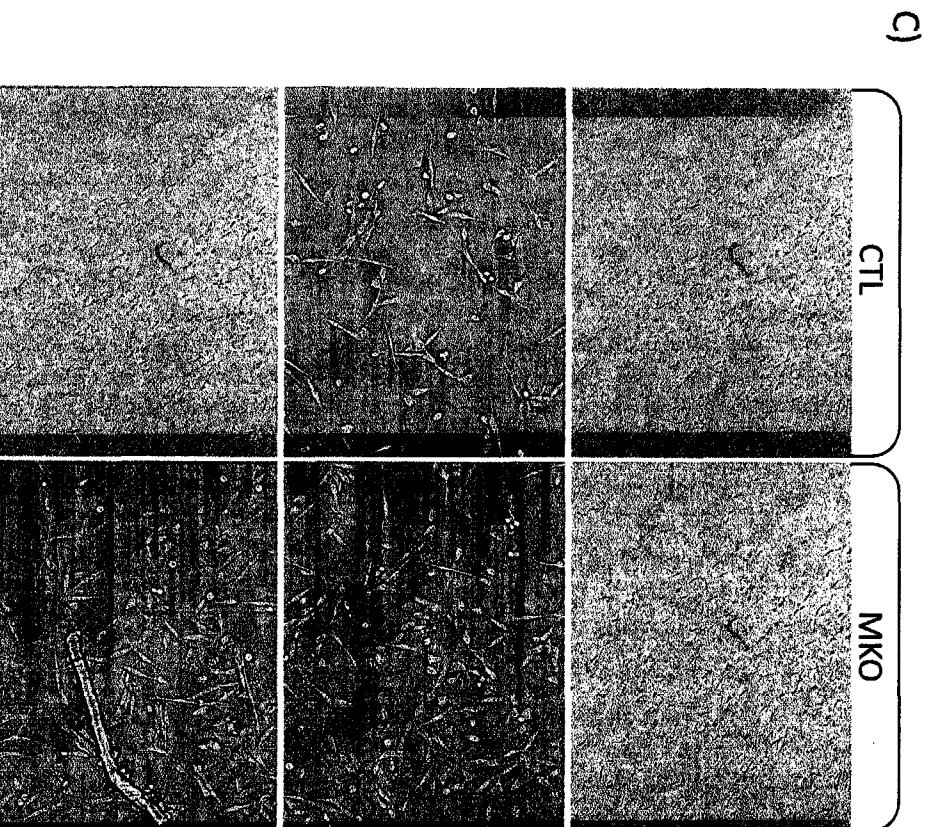
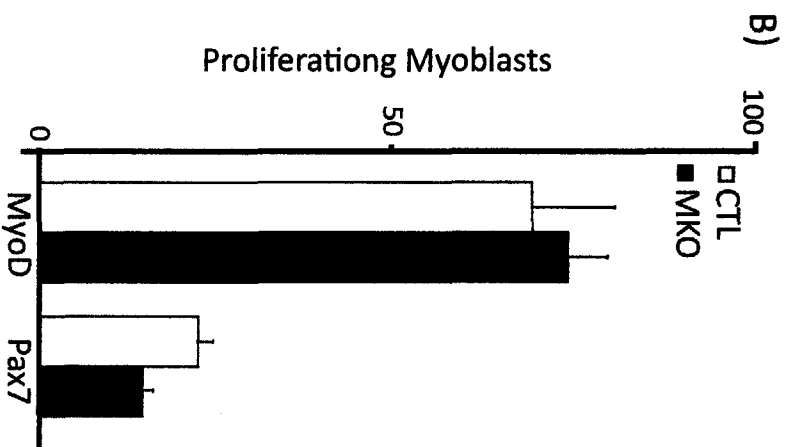
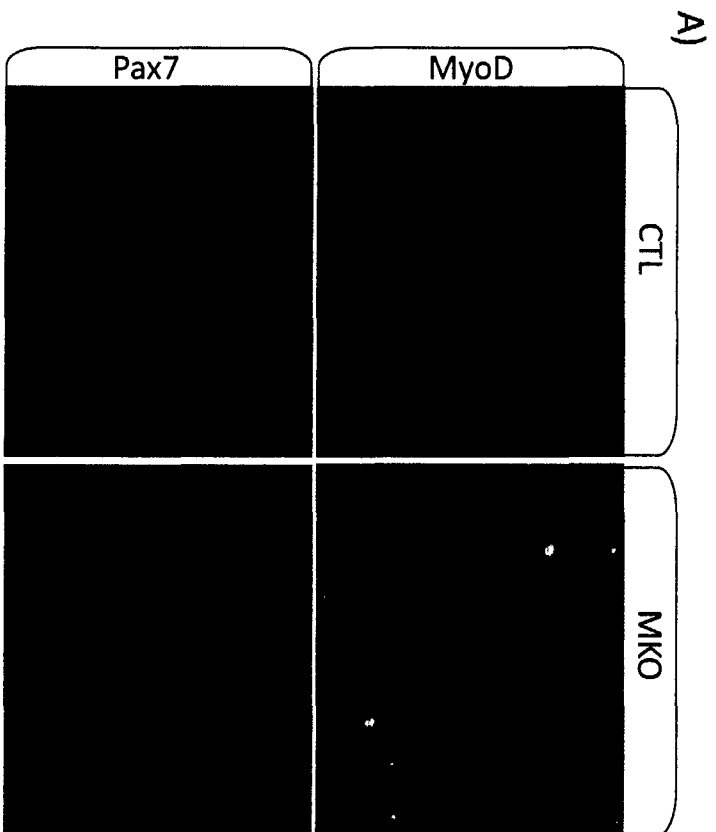


Figure 13. Decreased expression of the satellite cell marker, Pax7, in the absence of muscle-derived BDNF. A) Quantification of the relative levels of Pax7 mRNA in post-natal day 7 (P7), adult EDL, TA, and DIA muscles, and single fiber myoblast preparations (MB) from BDNF^{MKO} (MKO) and control litter mates (CTL). B) Relative levels of Pax7 mRNA in BDNF^{-/-} (KO) compared to control littermates at P1 (n=3). C) Quantification of Pax7 protein levels standardized to GAPDH (n=6, p<0.05). Error bars represent SEM.

Kuang et al, 2006). Our findings that Pax7 expression is significantly decreased in the absence of muscle-BDNF suggests that BDNF may play a role in regulating muscle satellite cell function and/or maintenance. In order to determine whether loss of muscle-derived BDNF compromised the integrity of the satellite cell pool, we examined proliferation and differentiation of satellite cell-derived myoblasts following single fiber isolation and culture. First, we performed immunofluorescent staining to detect MyoD and/or Pax7 following 5 days proliferation in order to ensure that BDNF^{MKO} cultures maintained muscle gene expression. Compared to control cultures, MyoD was expressed at normal levels, suggesting that in the absence of BDNF, satellite cell-derived myoblasts remained myogenic (Fig. 12). In line with transcript and protein quantifications (Fig. 13), the relative number of Pax7+ nuclei was decreased in BDNF^{MKO}-derived cultures compared to controls (Fig. 14A). Interestingly however, we also noted an increase in the number of MyoD+ cells in BDNF^{MKO} cultures (Fig. 14B and C).

To further investigate the proliferative properties of cultured BDNF^{MKO} myoblasts, we performed bromodeoxyuridine (BrdU) labelling over a period of 30 minutes growth to detect actively proliferating cells. In line with the above mentioned studies, we found an increase in the number of BrdU labelled nuclei associated with BDNF^{MKO} fibers, suggesting proliferation was enhanced in the absence of muscle BDNF (Fig. 15A and B, $p < 0.05$). We also examined the rate of cell cycle withdrawal following 24 hour exposure to differentiation medium. Upon induction of differentiation, we observed a greater number of BrdU labelled nuclei in BDNF^{MKO} cultures compared to controls (Fig. 15A and B). Furthermore, BDNF^{MKO} cultures exhibited delayed induction of several molecular markers of differentiation compared to controls, including reduced myogenin, p21, embryonic myosin heavy chain (embMyHC) and neonatal MyHC (Fig. 15C). Therefore, cell cycle exit and induction of molecular differentiation are delayed in the

Figure 14: Increased number of satellite cell derived myoblasts, and decreased percentage of Pax7+ nuclei in BDNF^{MKO} primary cultures A) Representative images of MyoD (green, top panels) and Pax7 (red, bottom panels) labelled myoblasts derived from control and BDNF^{MKO} (MKO) single fiber isolations. B) Quantification of the relative number of MyoD+ and Pax7+ myonuclei in single fiber-derived myoblast cultures following 5 days proliferation. C) Phase-contrast images of single-fiber myoblast cultures isolated from CTL and MKO muscles. D) Quantification of the number of single-fiber derived cells per myofiber following 5 days proliferation (n=6, p<0.05).



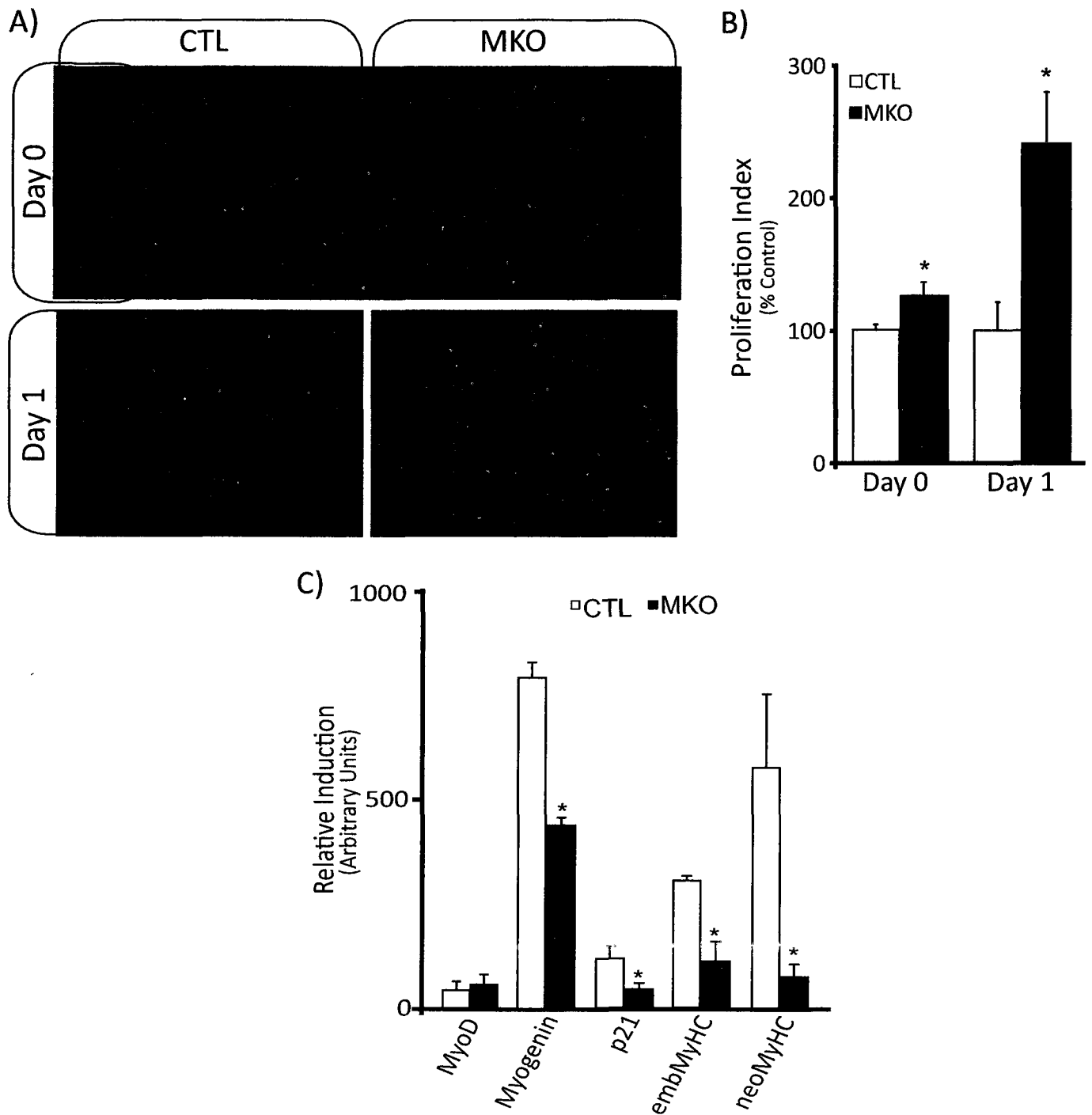


Figure 15. Abnormal satellite cell proliferation and delayed induction of genes involved in myogenic differentiation in single fiber myoblast preparations from BDNF^{MKO} mice. A) Representative BrdU labeling (green) of proliferating (Day 0, top panels) and early differentiating (Day 1, bottom panels) myoblasts isolated from BDNF^{MKO} (MKO) and control (CTL) litter mates. Images are counterstained with 4',6-diamidino-2-phenylindole (DAPI) in blue. B) Quantification of the ratio of BrdU labeled nuclei relative to the total number of nuclei per field of view (n=6, *p<0.05). C) Relative induction of MyoD, myogenin, p21, embryonic (emb) and neonatal (neo) myosin heavy chain (MyHC) transcripts compared to proliferating cultures following 1 day exposure to differentiation promoting medium (n=6 performed in triplicate, *p<0.05). Error bars represent SE.

absence of muscle BDNF. Again, MyoD expression was not changed, suggesting myogenic determination of satellite cell-derived myoblasts was not affected (Fig. 14A and Fig. 15C). Together these results suggest BDNF is required for early phases of myogenic differentiation, and that in the absence of BDNF, myogenic lineage progression is adversely affected.

To delineate whether myogenic lineage progression was temporarily delayed or terminally affected in BDNF^{MKO} cultures, and to examine the effects of BDNF depletion on later stages of myogenic differentiation, we examined myotube formation following 5 day exposure to differentiation medium. Compared to controls, BDNF^{MKO}-derived myotubes contained fewer myonuclei, and exhibited an increase in the number of unfused (mononuclear) myocytes expressing MyHC (Fig. 16A and B). Consequently, the average size of MyHC-expressing myotubes was significantly decreased (Fig. 16C, $p < 0.05$) and MyHC transcript was expressed at significantly lower levels than controls (Fig. 16D, $p < 0.01$). However, the majority of myotubes did express MyHC within 5 days, suggesting that terminal differentiation is not affected at the molecular level, but rather, there is a significant delay in the induction of molecular markers of differentiation. The observed reduction in myoblast fusion and formation of large multinucleated myotubes may thus be a direct consequence of delayed induction of molecular markers of differentiation. Alternatively, it may suggest that BDNF plays an additional role in fusion and growth of multinucleated myotubes.

Finally, to determine whether delayed differentiation was specifically due to BDNF-depletion, we attempted to rescue normal satellite cell function by addition of exogenous BDNF to culture media. Daily treatment with 20ng/ml of exogenous BDNF protein was sufficient to rescue myotube size (Fig. 17A and B, $p < 0.05$) and MyHC expression (Fig. 17A and C, $p < 0.01$) following 5 days differentiation of BDNF^{MKO} cultures. These results suggest BDNF protein is

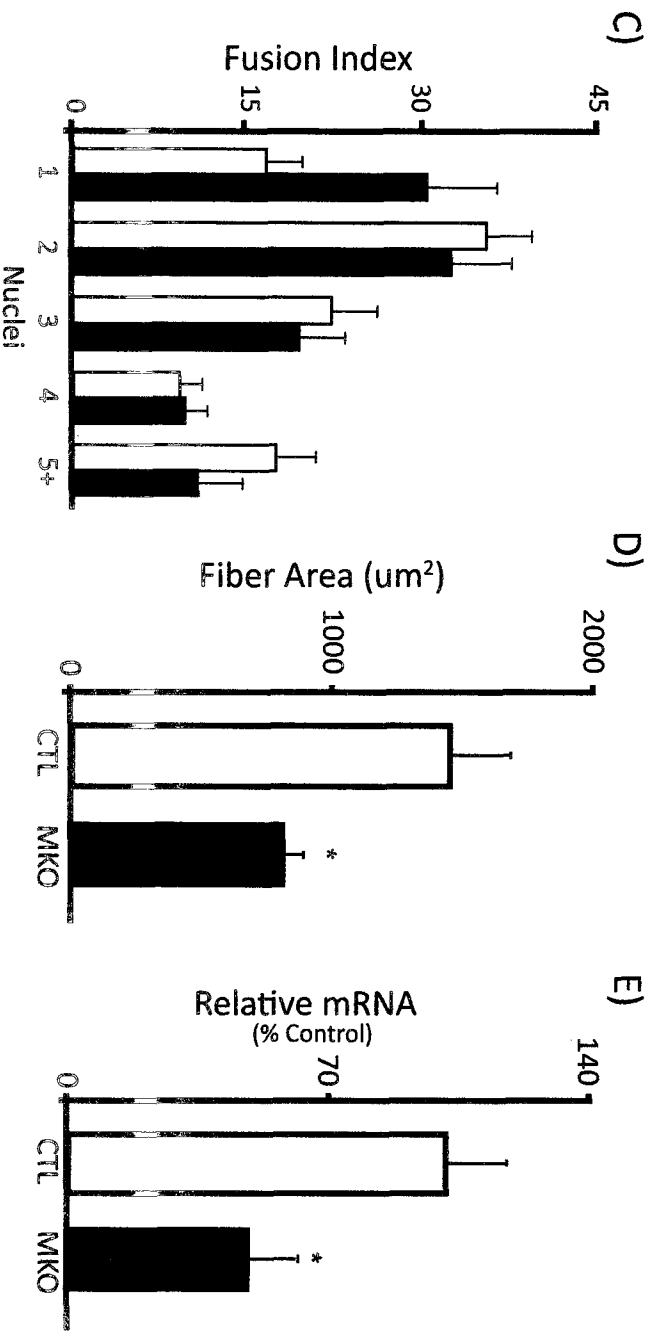
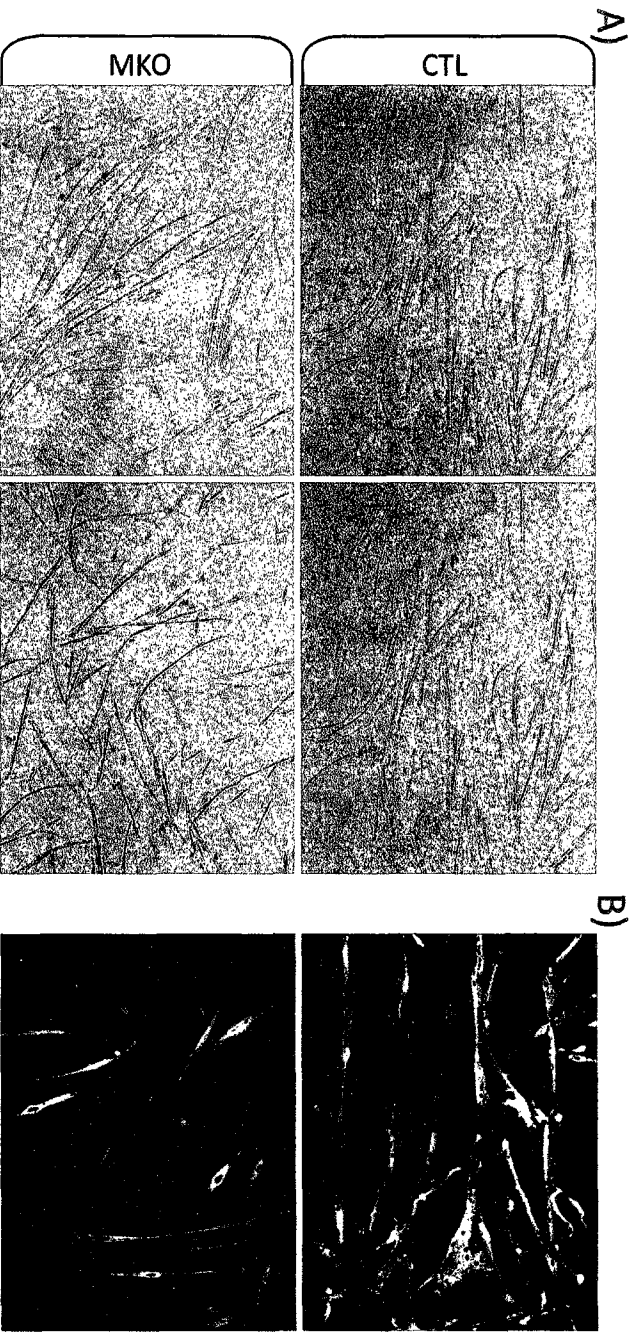


Figure 16. Decreased size and MYHC expression of BDNF^{MKO} derived myotubes. A) Representative phase contrast images of 5 day differentiated myotubes from BDNF^{MKO} (MKO) and control (CTL) cultures. **B)** Immunofluorescent labelling of total myosin heavy chain (MYHC- green) in CTL and MKO myotubes. **C)** Quantification of the number of nuclei per myotube following 5 day exposure to differentiation promoting medium in MKO and CTL cultures (n=3). **D)** Quantification of the average area of MYHC-positive myotubes (n=3, p<0.05). **E)** Relative MYHC mRNA levels standardized to GAPDH following 5 days differentiation (n=6 performed in triplicate, *p<0.05)

A)

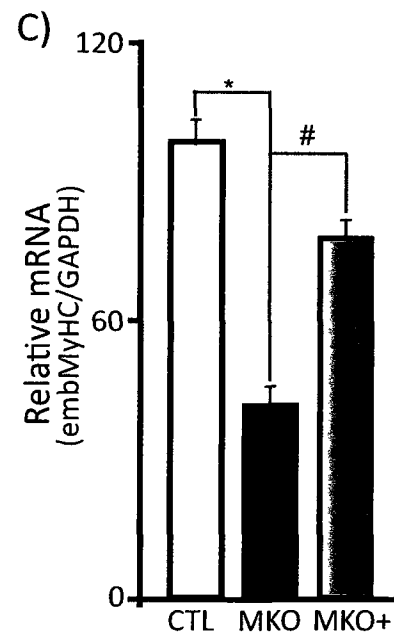
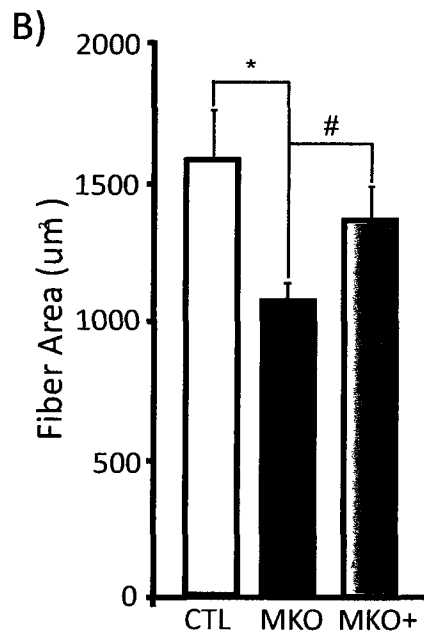
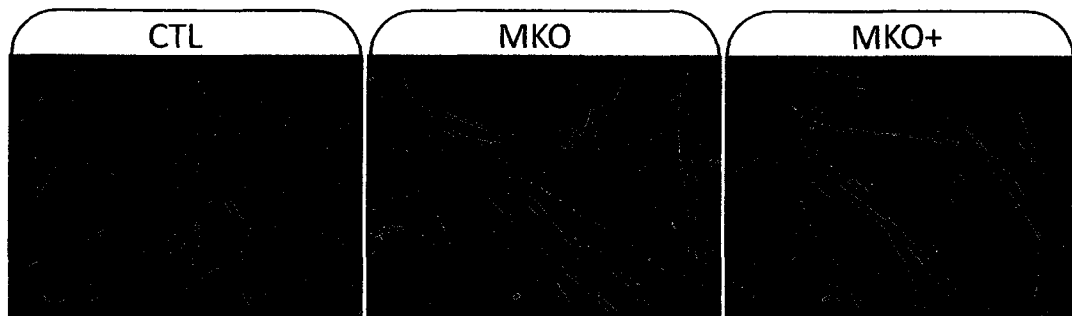


Figure 17. Decreased size and MyHC expression in BDNF-depleted myotubes is rescued by treatment with exogenous BDNF protein. A) Immunofluorescent labelling of total myosin heavy chain (MyHC) in control (CTL), BDNF^{MKO} (MKO), and MKO cultures treated with 20ng/ml of recombinant BDNF protein (MKO+) following 5 days differentiation. Counterstaining with 4',6-diamidino-2-phenylindole (DAPI) was performed. B) Quantification of the average area of MyHC-positive myotubes. C) Relative MyHC mRNA levels standardized to GAPDH after 5 days differentiation (n=6, * represents significant decrease compared to control, # significant increase compared to MKO, p<0.05)

required for normal myogenic differentiation of satellite cell-derived myoblasts, and shows that delayed differentiation of BDNF^{MKO} cultures was not the result of intrinsic changes to the satellite cell pool.

3.8 BDNF Expression During Cardiotoxin-Induced Muscle Regeneration

Following injury, muscle satellite cells become activated, proliferate, differentiate and fuse with each other, or with existing fibers to repair damaged the tissue. To determine whether muscle-derived BDNF is important for satellite cell function *in vivo*, we examined skeletal muscle regeneration in 5-8 week old BDNF^{MKO} mice. Unfortunately, these studies were not possible in the BDNF^{-/-} mouse due to early postnatal lethality of the complete BDNF knockout.

In order to determine whether satellite cell function and muscle regeneration was compromised in the absence of muscle BDNF, we performed time course studies on regenerating muscles following cardiotoxin (CTX) injury (Gramolini et al, 1999; Condrea, 1974). In control muscles, BDNF transcript levels were significantly upregulated within 2 days post-injury, followed by a gradual decrease over the 7 day time course (Fig. 18A, $p < 0.05$). In contrast, BDNF levels remained significantly reduced throughout early time points in regenerating BDNF^{MKO} muscle, reaching only 50% of uninjected control levels 0-2 days post-injury (Fig. 18A, $p < 0.05$). The disparity in BDNF levels was most apparent 2 days post-injection, when control BDNF levels were more than 3-fold higher than BDNF^{MKO} levels.

Interestingly however, BDNF expression increased during later time points in BDNF^{MKO} muscle, achieving control levels 5-7 days post-injury (Fig. 18A). Although we were surprised to find such high levels of BDNF in regenerating BDNF^{MKO} muscle, we hypothesized that BDNF

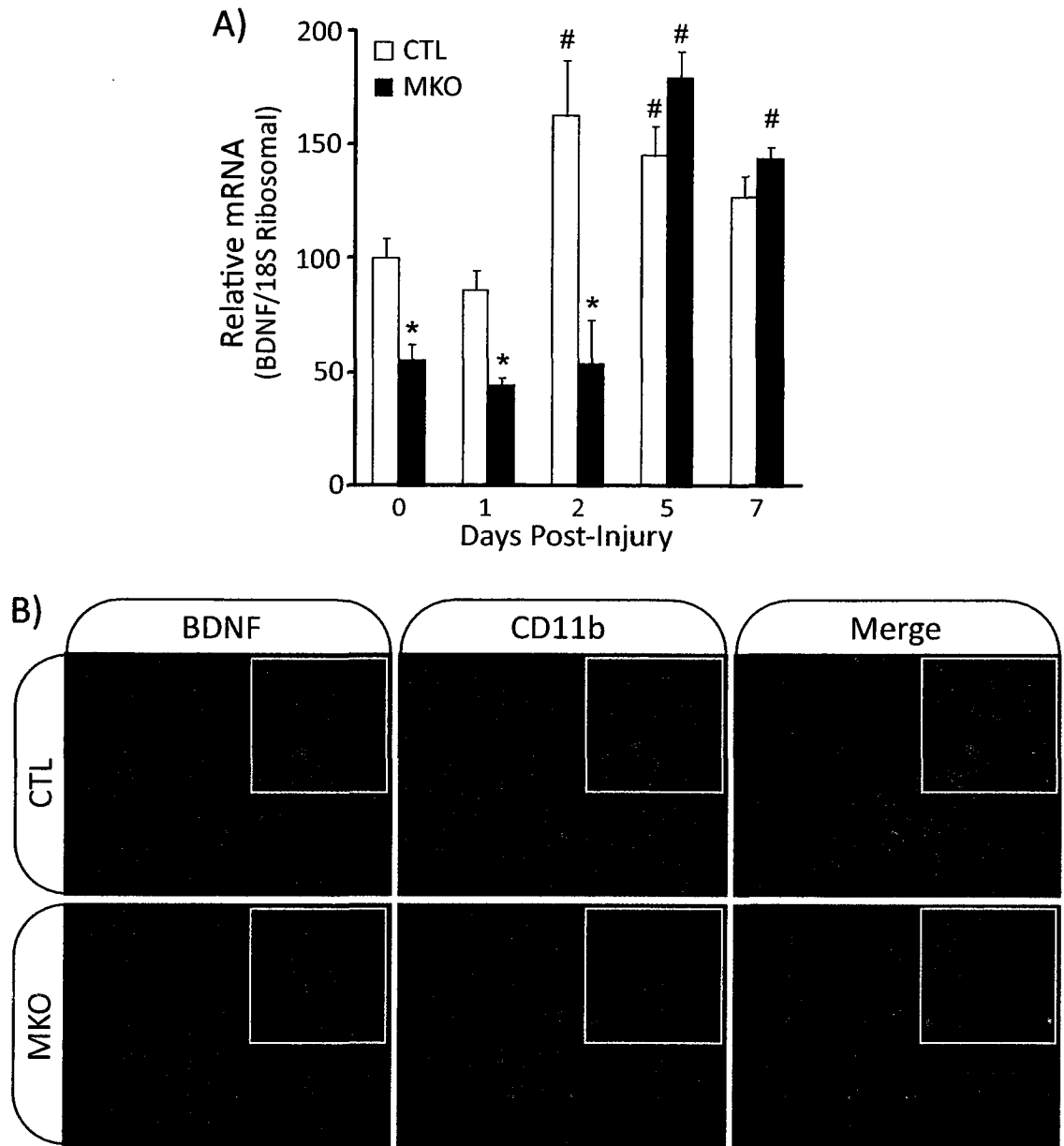


Figure 18. BDNF expression by CD11b-positive cells in regenerating skeletal muscle. A) Quantification of the relative levels of BDNF transcript in regenerating BDNF^{MKO} (MKO) and control (CTL) muscles at 0, 1, 2, 5, and 7 days post-cardiotoxin injection (n=6, * decrease, # increase compared to controls at day 0, p<0.05). B) Immunofluorescent labelling of BDNF (green) and CD11b (red) in regenerating TA muscles of MKO and CTL animals 5 days post-injury.

was secreted by resident non-muscle cells, and/or infiltrating immune cells following injury (see below, and Kerschensteiner et al, 1999; Vega et al, 2003). To examine the source of BDNF in regenerating BDNF^{MKO} muscle, we performed immunolabelling experiments on regenerating TA muscle sections 5 days post injury, when BDNF levels were at their peak. Because previous studies had shown that BDNF levels correlate with immune cell infiltration in a number of human neuropathies (Sobue et al, 1999), we examined BDNF localization with respect to the immune cell marker CD11b. BDNF staining colocalized with CD11b⁺ immune cells in both control and BDNF^{MKO} sections (Fig. 18B). Importantly however, control cross sections showed both BDNF⁺/CD11b⁺ and BDNF⁺/CD11b⁻ labelling, while KO sections showed exclusively BDNF⁺/CD11b⁺ labelling. The observation that BDNF labelled cells co-expressed CD11b in BDNF^{MKO} tissue indicates that BDNF expression is immune cell-derived in regenerating BDNF^{MKO} muscle. Conversely, BDNF⁺/CD11b⁻ labelling in control sections suggests that additional cell types express BDNF in response to muscle injury.

3.9 Regeneration is Delayed in the Absence of Muscle-Derived BDNF

Although BDNF transcript levels were eventually upregulated in injured BDNF^{MKO} muscle, expression levels did not reach control values until 5 days post-injection (Fig. 18A). Thus, BDNF levels were significantly reduced during early time regeneration time points. To determine whether delayed expression of BDNF adversely affected the early regenerative response to injury, we examined a number of parameters of myogenic differentiation. In agreement with our culture studies, we found that induction of several molecular markers of

regeneration, including Pax7, MyoD, myogenin and embMyHC was delayed in BDNF^{MKO} muscle 5 days post-injury (Fig. 19A-D, p<0.05).

We also performed histological examination of regenerating muscle 5 and 7 days post-injection. Newly formed fibers were quantified as the number of centrally nucleated fibers per field of view in regenerating cross-sections. Consistent with a decrease in regenerative markers, we observed 40% fewer regenerated fibers in BDNF^{MKO} muscle 5 days post-injury when compared to control littermates (Fig. 20A and B, p<0.05). Concomitantly, we observed a 25% increase in the number of mononuclear cells within regenerating BDNF^{MKO} compared to control littermates (Fig. 20A and C, p<0.05). Together, these findings suggest that in the absence of muscle-derived BDNF, early regeneration is delayed. In addition, our results suggest that non-muscle derived BDNF may play an important role in various aspects of skeletal muscle regeneration

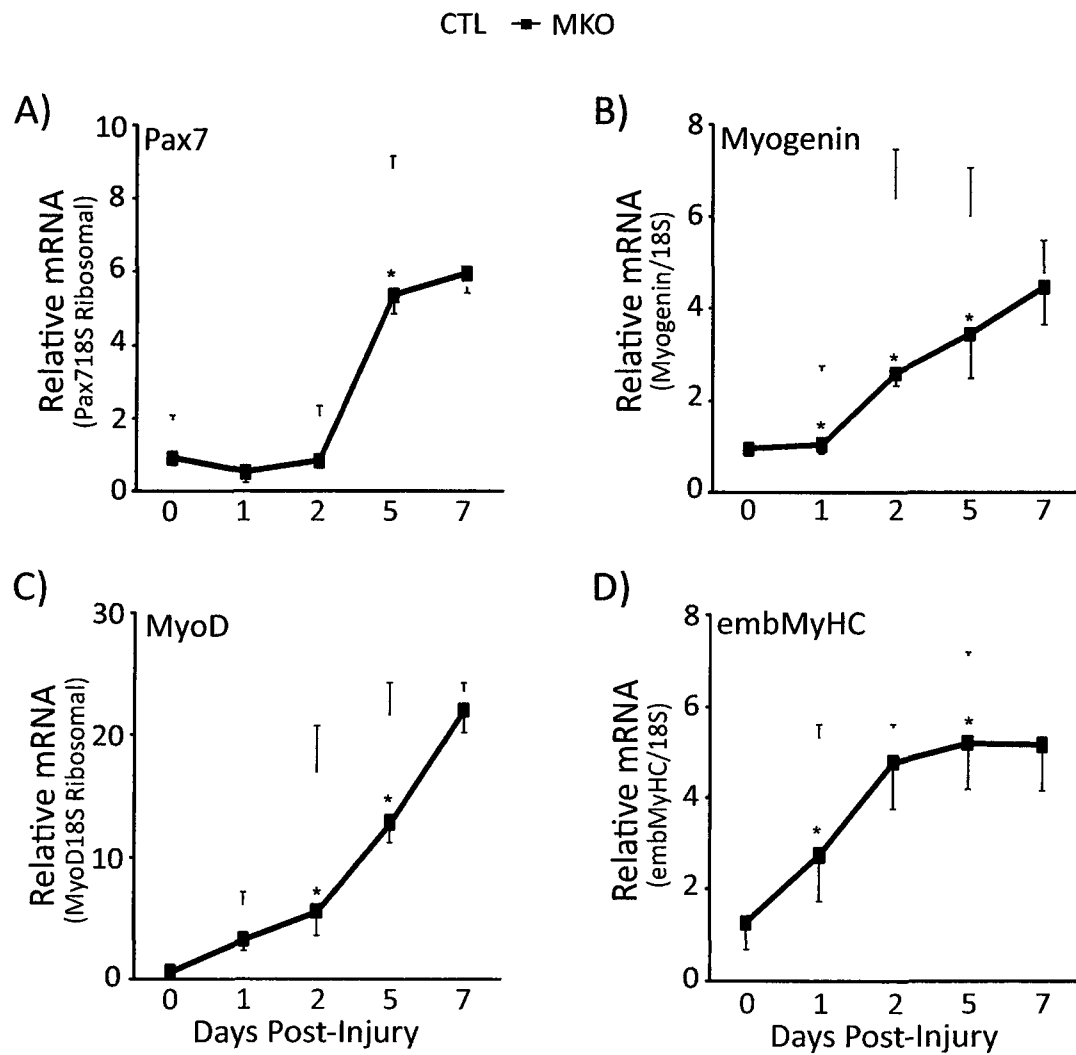


Figure 19. Delayed induction of molecular markers of regeneration in the absence of muscle-BDNF. A) Quantification of the relative levels of Pax7 B) Myogenin, C) MyoD, and D) embryonic myosin heavy chain (embMyHC) transcript levels in regenerating BDNF^{MKO} (MKO) and control (CTL) tibialis anterior muscles 0, 1, 2, 5, and 7 days following injury. All values are relative to control day 0 transcript levels (n=6, p<0.05). Error bars represent SEM

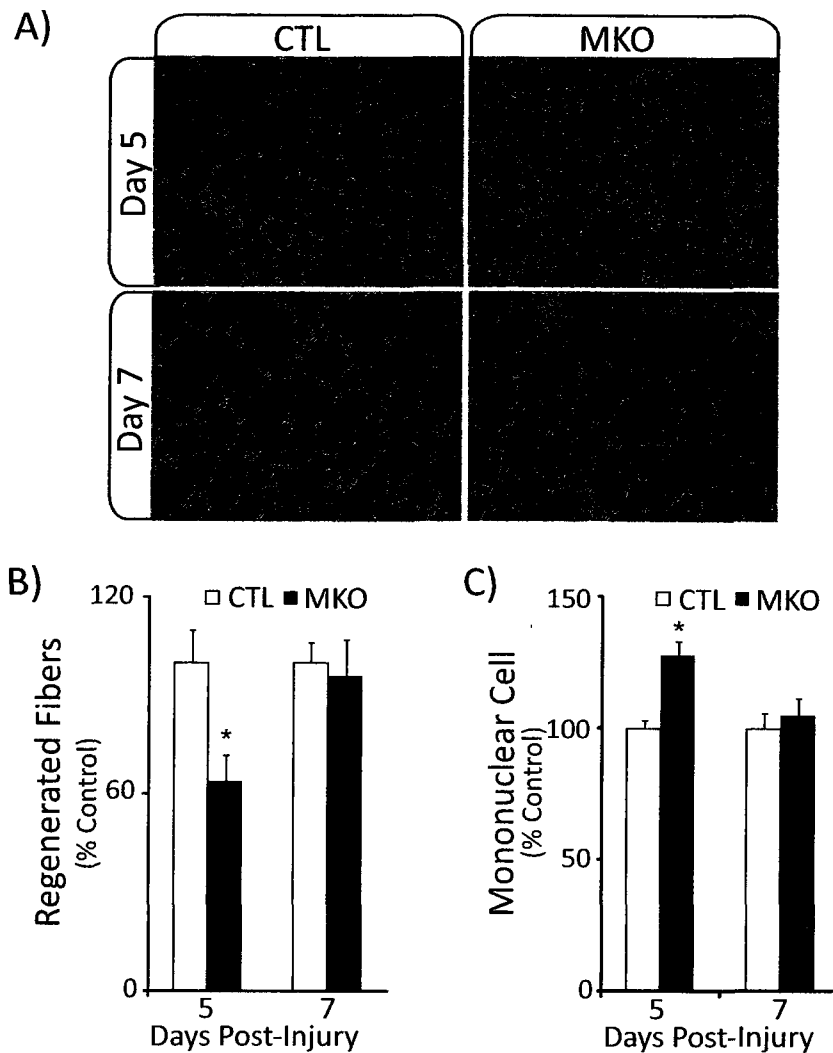


Figure 20. Delayed regeneration in the absence of muscle-BDNF. A) Representative H&E staining of regenerating TA muscles from CTL and MKO animals at days 5 (top panels) and 7 (bottom panels) post-injury. B) Quantification of the number of newly regenerated (centrally nucleated) fibers in CTL and MKO muscles at 5 and 7 days post-injury. C) Relative number of mononuclear cells within regenerating MKO and CTL muscles at 5 and 7 days post-injury. All values are relative to control levels (n=3, *p<0.05). Error bars represent SEM

CHAPTER 4

Discussion

4. Discussion

In the present study, we examined the role of BDNF in postnatal muscle development using BDNF^{-/-} mice and transgenic mice in which BDNF was specifically depleted from skeletal muscle cells. The muscle-specific BDNF knockout mouse (BDNF^{MKO}) circumvents the problems of early post-natal lethality and breeding issues presented by BDNF^{-/-} mice, and allows examination of the specific role of muscle-derived BDNF in skeletal muscle development, maintenance and regeneration. In characterizing the BDNF^{MKO} mouse, we demonstrate that muscle-derived BDNF regulates proliferation and differentiation of satellite cell primary cultures and muscle regeneration *in vivo*. These findings demonstrate an important role for BDNF in regulating satellite cell function and muscle regeneration, particularly during early stages of myogenic differentiation.

4.1 Generation and Characterization of the Muscle-Specific BDNF Knockout Mouse

Muscle-derived BDNF has classically been described as a retrograde trophic factor responsible for supporting the survival and maintenance of innervating motor neurons throughout their lifespan (Purves, 1988; Davies, 1996). However, recent studies in our laboratory have shown that in adult skeletal muscle, BDNF expression is not localized to the neuromuscular junction, and is instead expressed in muscle satellite cells (Mousavi and Jasmin, 2006). In the same study, siRNA knockdown experiments suggested a role for BDNF in regulating differentiation of myoblasts in culture (Mousavi and Jasmin, 2006). Our present studies confirm these findings, and suggest a physiologically significant *in vivo* role for BDNF in regulating satellite cell function and muscle regeneration.

In our initial characterization of the BDNF^{MKO} mouse, we found that BDNF was depleted by 50-60% in P7 and adult hindlimb muscles. However, close examination of single fiber preparations, where resident non-muscle cells are removed, confirmed that BDNF was depleted by more than 85% in muscle fibers and associated satellite cells. It is important to note that approximately 15% of control BDNF levels remained within our satellite cell preparations. Although this could indicate low levels of contamination within our culture preparations, it might represent a population of satellite cells that do not express Myf5, and thus do not lose BDNF expression in the BDNF^{MKO} mouse. This Myf5-negative population is suggested to represent 13±4% of all Pax7 positive satellite cells, and thus correlates well with the remaining BDNF detected in primary cultures (Kuang et al, 2006). Furthermore, our results suggest a portion of the BDNF detected in whole muscle preparations is derived from resident non-muscle cells within the muscle compartment. Specifically, we found that ~50-60% of BDNF detected in whole muscle preparations is derived from Myf5-expressing muscle cells while another ~15% is derived from muscle cells that do not express Myf5. As such, our results suggest that 30-35% of detectable BDNF is derived from non-muscle cells within the muscle compartment. These findings are not entirely surprising, as the adult muscle compartment is composed of several cell types, including endothelial cells, neurons, Schwann cells and immune cells, all of which have been shown to express and secrete BDNF (Kerschensteiner et al, 1999; Vega et al, 2003; Kermani and Hempstead, 2007; reviewed; Gordon, 2009). These findings were further supported in our regeneration studies, where BDNF staining co-localized with CD11b+ immune cells. Thus, because BDNF is specifically depleted from Myf5-expressing cells, and because a portion of the cells within the muscle compartment do not express Myf5, it becomes important to consider the fact that low levels of secreted BDNF are available to BDNF^{MKO} muscle cells.

Because p75^{NTR} and TrkB receptor levels are not significantly different from control levels, some BDNF signalling may persist in BDNF^{MKO} muscle. As such, the phenotype of BDNF^{MKO} muscle should be considered less severe than it would be in the complete absence of BDNF.

4.2 The Role of BDNF in Muscle Fiber Type Determination

Although our results suggest a novel role for BDNF in regulating myogenic differentiation and satellite cell function, it is important to recognize that 50 years of research also suggests role for BDNF in establishment and maintenance of the neuromuscular junction (reviewed; Pitts et al, 2006). In accordance with this more classical role, we detected a significant decrease in expression of MyHC IIB in BDNF^{MKO} muscle, suggesting BDNF promotes expression of fast glycolytic MyHC IIB. This is in contrast to previously published data on NT-4/5, which has been shown to promote slow oxidative MyHC I fiber type transformation during development (Carrasco and English, 2004). In these studies, intramuscular injection of human recombinant NT-4/5 over 4-6 weeks resulted in increased expression of slow type I MyHC in neonatal rats. In the same study, addition of exogenous BDNF did not promote MyHC I expression. This fits well with our findings that although MyHC IIB was decreased in the absence of muscle BDNF, MyHC I was not affected (Carrasco and English, 2004). Together, these results suggest that although NT-4/5 and BDNF bind the same receptors (TrkB and p75^{NTR}), they play opposing roles in muscle fiber type specification. This finding is coherent with the idea that neurotrophins can play different roles depending on the cellular/physiological context, as well as the availability of putative receptors (reviewed; Richardt, 2006).

Although the precise link between neurotrophins and muscle fiber type is unclear, there are many possible mechanisms by which BDNF may influence MyHC expression. First, depletion of muscle-BDNF may result in intrinsic defects in muscle gene expression. For example, BDNF signals through p75^{NTR} and/or TrkB receptors which can activate a number of downstream signalling cascades including Ras/ERK, PI3/Akt, NF-κB, JNK or PLC-γ pathways. These pathways have been implicated in regulating physiological properties of skeletal muscle, and in the absence of muscle-BDNF, decreased signalling may result in repression or inactivation of pathways involved in promoting expression of MyHC IIB. For example, in a recent study by Shi and colleagues (2008), activation of the ERK signalling pathway was shown to promote expression of fast MyHC isoforms while repressing expression of slow MyHC. In the same study, inhibition of ERK activation resulted in decreased expression of fast MyHC isoforms in vitro (Shi et al, 2008). Because BDNF-mediated activation of TrkB signalling is known to activate the ERK pathway, it is possible that depletion of BDNF results in decreased ERK signalling within the muscle compartment, and subsequently, downregulation of MyHC IIB expression. However, expression of TrkB in skeletal muscle is controversial, such that these pathways may only be relevant very early in embryonic development (Lomen-Hoerth and Shooter 1995; Ip et al, 2001).

Alternatively, changes in MyHC expression may be the result of abnormal innervation of BDNF-depleted muscle. MyHC expression patterns depend heavily on electrochemical stimulation by innervating motor neurons (reviewed; Talmadge, 2000). In NT-4/5 knockout mice, disassembly of the neuromuscular junction (NMJ) has been described in slow soleus muscles, suggesting NT-4/5 supports the formation or maintenance of NMJs between innervating motor neurons and fibers destined to express MyHC I (Bellurado et al, 2001). NMJ disassembly

results in abnormal innervation of slow fibers and subsequently, decreased fatigue resistance in NT-4/5 knockouts. Similar findings were observed upon disruption of TrkB-mediated signalling (Gonzalez et al, 1999). As such, we hypothesized that changes in MyHC composition within BDNF-depleted skeletal muscle may be an indirect effect of abnormal innervation of IIB fibers. In order to examine this possibility, we examined NMJ morphology on single fiber preparations derived from extensor digitorum longus (EDL) muscles, which express predominantly (60-70%) fast-twitch MyHC IIB. Our preliminary data suggest BDNF is indeed required for normal formation and/or maintenance of these NMJs, as BDNF^{MKO} fibers exhibited a high incidence of abnormal NMJ morphologies (Fig. 11). Importantly, junctional abnormalities were apparent in fiber bundles derived from fast-twitch EDL, but not in fibers derived from predominantly slow-twitch soleus (SOL) or tibialis anterior (TA) muscles. Together, these preliminary findings suggest that BDNF is required to support a specific subpopulation of motor neurons innervating fast-twitch (MyHC IIB) fibers. These findings are in accordance with previously published data showing that BDNF, together with ciliary-derived neurotrophic factor (CNTF), supports a subpopulation of motor neurons responsible for innervating type IIB muscle fibers during neonatal development of the neuromuscular system (Mousavi et al, 2004). Although further characterization of the junctional abnormalities associated with loss of muscle-BDNF is necessary, our preliminary studies suggest that loss of muscle-BDNF results in abnormal innervation of MyHC IIB fibers, and that this physiological change to the muscle fiber induces changes in the MyHC expression profile.

4.3 The Role of Muscle-Derived BDNF in Postnatal Muscle Development

Myogenic differentiation is a highly coordinated event involving ordered progression through various states of cellular and molecular differentiation (reviewed; Charge and Rudnicki, 2004). It involves commitment to the myogenic lineage through expression of ‘early’ MRFs (Myf5 and MyoD), which in turn promote proliferative expansion in response to mitogenic signals from the environment (Braun et al, 1992; Rudnicki et al, 1992; Rudnicki et al, 1993; 1993; Megeney et al, 1996; Yablonka-Reuveni et al, 1999). Differentiation ensues with the upregulation of ‘late’ MRFs (myogenin and MRF4), followed by irreversible cell cycle withdrawal, induction of expression of contractile proteins, and myoblast fusion to form multinucleated myotubes (Hasty et al, 1993; Nabeshima et al, 1993; Wang and Jaenisch, 1997). In the present study, we showed that transgenic knockdown of muscle-BDNF resulted in decreased expression of the satellite cell marker Pax7 in adult skeletal muscle. In order to determine whether this decrease was associated with a change in the myogenic properties of BDNF-depleted cells, we isolated single fibers and their associated satellite cells and cultured them in conditions that promoted growth and differentiation of myoblasts. In this system, it became clear that muscle-derived BDNF is important for normal satellite cell function. Although Pax7 expression levels were decreased compared to controls, the number of satellite cell-derived myoblasts was similar in culture, as were expression levels of myogenic determination gene MyoD, suggesting that commitment of BDNF^{MKO} satellite cells to the myogenic lineage was not affected. However, all subsequent steps involved in myogenic lineage progression, including expression of ‘late’ MRFs, p21 and developmental MyHC isoforms appear to require muscle-BDNF for proper induction. Addition of exogenous BDNF protein was sufficient to rescue both myotube size and MyHC induction, suggesting BDNF itself is required for normal satellite cell

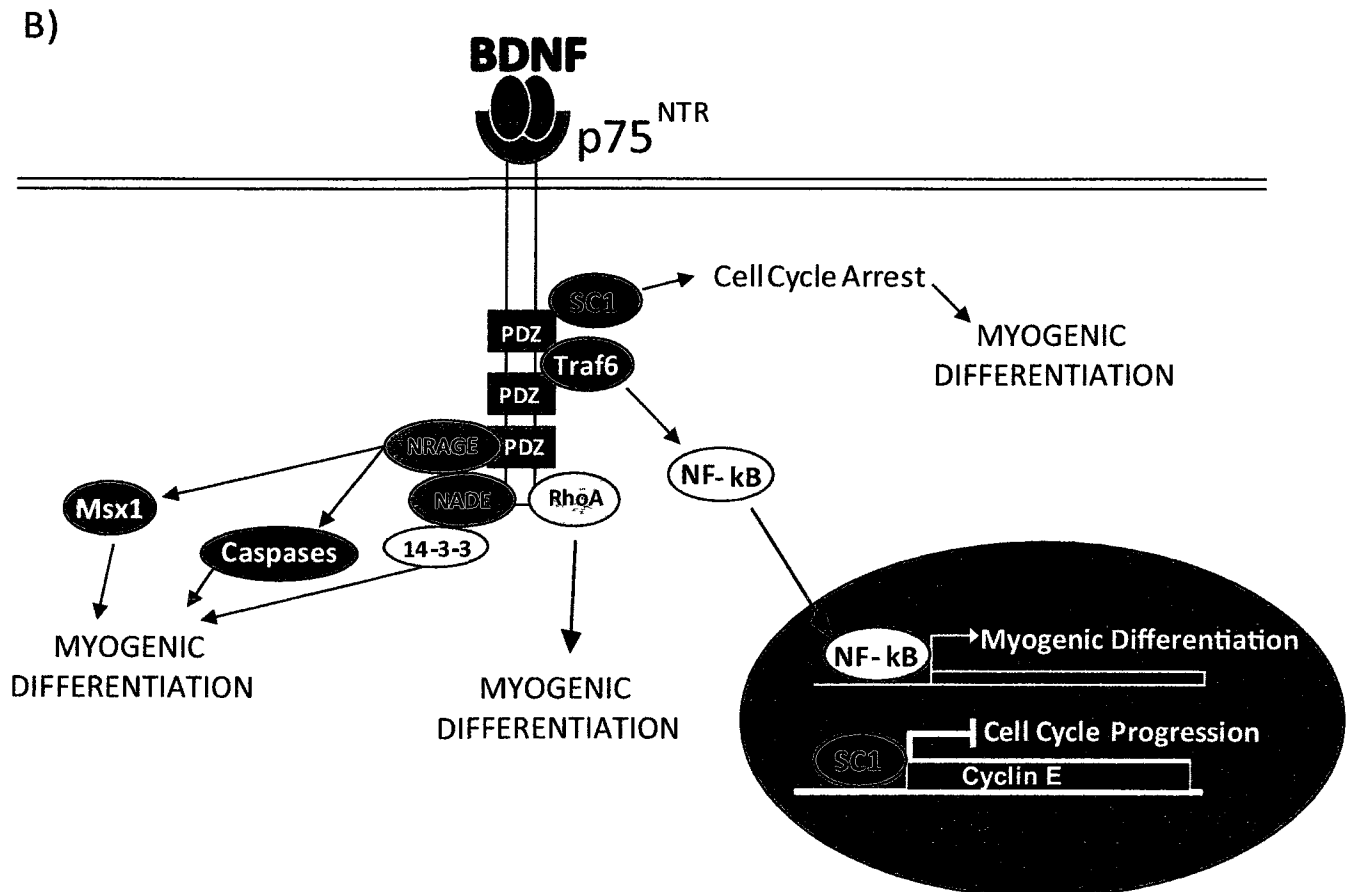
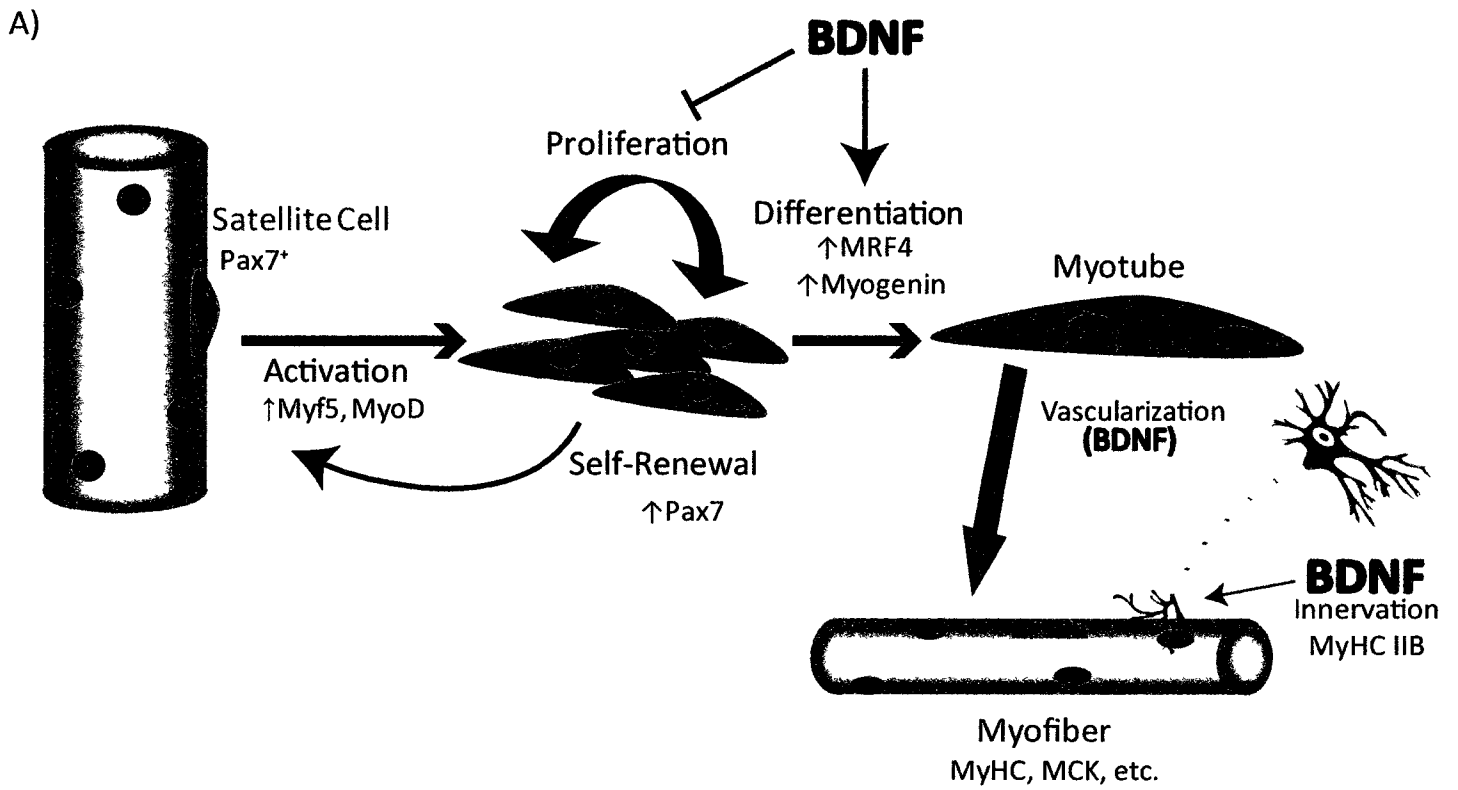
function. Furthermore, these results suggest depletion of muscle-BDNF does not alter the intrinsic ability of muscle satellite cells to undergo normal differentiation given exposure to appropriate extracellular stimuli. Therefore, our results strongly suggest BDNF secreted from muscle cells is capable of autocrine signalling within the muscle compartment, and that downstream signalling cascades promote the expression of genes involved in myogenic lineage progression and ultimately, differentiation and myotube formation (Fig. 21A).

4.4 The Role of BDNF in Skeletal Muscle Regeneration

Similar results were observed *in vivo* upon activation of the myogenic program during regeneration. Following cardiotoxin injury, early regeneration was delayed in BDNF^{MKO} muscles compared to controls, with delayed induction of molecular and histological signatures of repair. Interestingly however, normal regenerative capacity was completely restored within 7 days. Recovery of normal regeneration occurred at a point when BDNF expression had increased to control levels in BDNF^{MKO} muscle as a result of secretion from infiltrating CD11b+ immune cells. As such, BDNF secretion from non-muscle cells appears to comprise the compensatory mechanism by which normal myogenic potential is restored to BDNF^{MKO} tissue. In agreement with our culture studies, the observed delay in regeneration appears to be in response to a specific requirement for BDNF during myogenic differentiation rather than an intrinsic defect in BDNF^{MKO} muscle satellite cells. Given that BDNF receptor levels are unchanged, secreted BDNF would be capable of normal signalling within the muscle compartment, allowing for recovery of normal regeneration. If this is the case, BDNF may play a similar role to other inflammatory cytokines (TGF- β , IL-6) in regulating muscle regeneration (reviewed; Tidball,

Figure 21: Proposed model of the role of BDNF in postnatal muscle growth and regeneration.

A) Our results in the BDNF^{MKO} mouse suggest that BDNF plays a role in regulating myogenic differentiation at various stages of myogenic lineage progression. Specifically, our studies in primary culture and in vivo regeneration suggest that BDNF is required for normal cell cycle exit, regulation of proliferation and induction of early molecular markers of differentiation. Furthermore, our myosin heavy chain (MyHC) expression profiling studies suggest that BDNF is also required for normal MyHC IIB expression, possibly by supporting a subpopulation of motor neurons that support IIB fibers. B) BDNF signalling through the low affinity p75 neurotrophin receptor (p75^{NTR}) is hypothesized to mediate myogenic lineage progression through multiple downstream signalling cascades.



2005). Furthermore, it becomes apparent that immune-cell derived BDNF is an important source of BDNF during muscle injury and repair. Similar findings have been reported in human peripheral neuropathies, in which BDNF expression was proportional to the extent of invasion by T cells and macrophages rather than to the specific disease pathology (Sobue et al, 1998).

4.5 BDNF Signalling in Myogenesis

Although further studies are required to elucidate the precise molecular pathways involved in BDNF-mediated progression of myogenic differentiation, converging lines of evidence suggest that signalling through the low affinity p75 neurotrophin receptor (p75^{NTR}) plays an important regulatory role. First, TrkB expression is controversial in skeletal muscle. While the majority of studies do not detect TrkB in adult skeletal muscle, a few have reported TrkB expression at neuromuscular junction (reviewed; Pitts et al, 2006). This is in contrast to p75^{NTR}, which is readily detectable in embryonic and adult skeletal muscle (Yan and Johnson, 1988; Lomen-Hoerth and Shooter, 1995). Furthermore, p75^{NTR} can mediate a number of important regulatory signalling cascades depending on the availability of ligands, co-receptors, and downstream signalling molecules. Several targets of p75^{NTR} have been implicated in neuronal differentiation, and a number of studies have suggested these targets play a similar role in skeletal muscle (Seidl et al, 1998; Reddypalli et al, 2005; Deponi, 2009). Of particular interest to our study are recent findings by Deponi and colleagues showing that p75^{NTR} signalling is required for normal satellite cell function and muscle regeneration (Deponi et al, 2009). In their study, inhibition of NGF-mediated p75^{NTR} signalling resulted in increased accumulation of RhoA-GTP, which negatively affected myoblast fusion and cytoskeletal

organization (Deponi et al, 2009). These findings were recapitulated *in vivo* during muscle regeneration, where injection of an NGF-competing peptide, or myoblasts infected with constitutively active form of RhoA resulted in decreased regeneration following cardiotoxin injury (Deponi et al, 2009). However, these studies did not detect a change in muscle-specific gene expression (MRFs, MyHC, etc.), suggesting additional or other p75^{NTR}-mediated signalling pathways are affected in the BDNF^{MKO} mouse.

A number of additional p75^{NTR} mediated signalling pathways have been implicated in cell cycle arrest and differentiation (Fig. 21B). For example, one target of p75^{NTR}, Schwann cell factor 1 (SC1), is a known transcriptional repressor involved in cell cycle arrest through repression of the pro-mitotic gene *cyclin E* (Chittka et al, 2004). Overexpression of cyclin E results in enhanced proliferation, and has been associated with various human tumours, including rhabdosarcomas. Loss of BDNF-p75^{NTR}-SC1 mediated cyclin E repression may therefore explain the excessive BrdU-labelling detected in BDNF^{MKO} cultures (Jin et al, 2007). Furthermore, p75^{NTR} regulates a number of pathways involved in death and differentiation, including NRAGE which has been shown to interact with Necdin and the Msx2 homeodomain protein to promote differentiation of C2C12 myoblasts (Kuwajima et al, 2004). NRAGE activation, can also lead to activation of caspases, including caspase 3, which is necessary for myogenic differentiation of C2C12 myoblasts (Fernando et al, 2002). Alternatively, p75^{NTR} mediated activation of the NF- κ B pathway may be adversely affected in the absence of BDNF. Numerous studies have shown that NF- κ B activity is necessary for myogenic differentiation, and myoblasts lacking NF- κ B activity display defects in cell proliferation and cell cycle exit upon induction of differentiation (Guttridge et al, 1999). These findings are intriguingly similar to our results in BDNF-depleted myoblasts, and may represent the link between BDNF and myogenic differentiation.

Previous studies in our laboratory showed that siRNA-mediated depletion of BDNF resulted in precocious differentiation of rat L6 myoblasts (Mousavi and Jasmin, 2006). Based on these findings, we had expected to see accelerated differentiation of BDNF^{MKO} myoblasts in culture and during regeneration *in vivo*. Instead, we found that in the absence of muscle-BDNF, both myogenic differentiation of isolated satellite cells and regeneration were delayed. Thus, our more recent findings suggest that rather than playing an inhibitory role, BDNF is required for normal myogenic differentiation, specifically during early myogenic lineage progression. There are a number of possible causes for the differences observed between these studies. First, there are intrinsic differences in the properties of immortalized cells lines and transgenic animals. Cell lines, such as C2C12 and L6 myoblasts, mimic to a limited extent the physiological and molecular properties of primary cultures, however the number of interacting factors that can play a role *in vivo* is profoundly different from what is available in culture systems. Furthermore, neurotrophin protein and receptor levels are vastly different between cell lines and compared to primary cultures and muscle preparations (Seidl et al, 1998; Rende et al, 2000; Reddypalli et al, 2005; Mousavi and Jasmin, 2006). As such, signal transduction can differ depending on the cellular context. Second, the developmental timing of BDNF depletion is vastly different between the two studies. In our initial culture studies, BDNF was depleted from myoblasts that had already undergone normal myogenic specification, and thus initial programming was unaffected. In the BDNF^{MKO} mouse, BDNF is depleted from myogenic precursors very early in myogenic lineage commitment (embryonic day 8; Tajbakhsh et al, 1997). These differences, in conjunction with the different mechanisms used to deplete BDNF expression (siRNA transfection vs. transgenic knockout) could result in altered response of satellite cells to signals that promote growth and/or differentiation.

Given our present findings, it becomes important to consider that many features of embryonic muscle development are recapitulated during muscle regeneration, with similar changes in muscle gene expression, physiological properties, and functional characteristics. In BDNF^{MKO} muscle, Pax7 expression is decreased, satellite cell differentiation is defective, and regeneration is delayed. Together, these findings clearly show that BDNF is required for normal satellite cell function, and suggest that complete ablation of BDNF, if it did not result in early death due to severe neuronal deficits, would considerably exacerbate the observed muscle phenotype. Because regeneration is delayed in BDNF^{MKO} muscle, but recovers upon exposure to sufficient levels of BDNF, it is possible that during early postnatal growth, myogenesis is adversely affected in our mouse model. For example, Pax7^{-/-} mice lack satellite cells and fail to grow postnatally, resulting in death by ~2 weeks (Seale et al, 2000). Considering that satellite cell function is impaired in the BDNF^{MKO} mouse, we could expect to see similar deficiencies in postnatal growth and survival. However, because resident non-muscle cells can secrete BDNF, and because BDNF receptor expression is not affected, it appears likely that normal muscle development ensues, resulting in the subtle phenotype of the BDNF^{MKO} mouse that we observed postnatally.

4.6 Conclusions and Implications

Although the neurotrophic hypothesis has survived over 50 years of scrutiny, accumulating evidence suggests that neurotrophins may play a more widespread physiological role in non-neuronal tissue than originally believed. The observation that neurotrophins and their corresponding receptors are expressed in non-neuronal tissues, including skeletal muscle, has

expanded the field and led to a number of new and exciting questions (Lai and Ip, 2003; Sheard *et al.*, 2002; Yamamoto *et al.*, 1996). Recent studies of neurotrophin function within the skeletal muscle compartment suggest a pleiotropic role in regulating myogenic differentiation during postnatal muscle development and differentiation, and during formation and maintenance of the neuromuscular junction (Seidl *et al.*, 1998; Reddypalli *et al.*, 2005; Deponi, 2009). Our studies support this hypothesis, demonstrating a clear role for BDNF in regulating myogenic differentiation, while suggesting an additional role in establishment and/or maintenance of the neuromuscular junction.

Regulation and maintenance of the satellite cell pool is critical to ensure proper regeneration and repair of adult skeletal muscle. In disease states such as Duchenne muscular dystrophy (DMD), where muscles undergo consecutive rounds of regeneration/degeneration, the satellite cell pool is eventually exhausted (Jejurikar and Kuzon, 2003; Blau *et al.*, 1983). Decreased satellite cell activity and insufficient regeneration leads to replacement of muscle fibers with connective tissue and fatty deposits (fibrosis), and contributes to early death of the patient (DiMario *et al.*, 1991). Prolonging the regenerative capacity of satellite cells in DMD muscle might alleviate or delay weakening of skeletal muscles. Given the experimental evidence implicating BDNF in myogenic differentiation, it becomes important to examine the functional significance of BDNF in myopathic conditions, and to determine whether manipulation of BDNF levels can stimulate muscle regeneration. Understanding the underlying factors that influence regenerative capacity of skeletal muscle might someday result in the development of therapeutic interventions to prolong and improve the quality of life for individuals living with muscle degenerative diseases.

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