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CHARACTERIZATION OF THE ROLES OF MUSCLE-SYNTHESIZED BRAIN- DERIVED NEUROTROPHIC FACTOR AND PRESYNAPTIC TYROSINE RECEPTOR KINASE B IN MOTOR NEURON AXONAL TRANSPORT

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CHARACTERIZATION OF THE ROLES OF MUSCLE-SYNTHESIZED BRAIN- 
DERIVED NEUROTROPHIC FACTOR AND PRESYNAPTIC TYROSINE 
RECEPTOR KINASE B IN MOTOR NEURON AXONAL TRANSPORT

By

Luke A. VanOsdol

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CHARACTERIZATION OF THE ROLES OF MUSCLE-SYNTHESIZED BRAIN-DERIVED NEUROTROPHIC FACTOR AND PRESYNAPTIC TYROSINE RECEPTOR KINASE B IN MOTOR NEURON AXONAL TRANSPORT

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ABSTRACT

CHARACTERIZATION OF THE ROLES OF MUSCLE-SYNTHESIZED BRAIN-DERIVED NEUROTROPIC FACTOR AND PRESYNAPTIC TYROSINE RECEPTOR KINASE B IN MOTOR NEURON AXONAL TRANSPORT

By

Luke A. VanOsdol

Neurotrophins, such as brain-derived neurotrophic factor (BDNF), are small, diffusible proteins essential for the development, survival, function, and plasticity of neurons. Neurotrophin actions are primarily mediated by the tyrosine receptor kinase (Trk) family of receptors (Patapoutian and Reichardt, 2001). BDNF binding to its receptor, TrkB, results in autophosphorylation of tyrosine residues on the cytoplasmic domain, leading to the activated form, phosphorylated-TrkB (p-TrkB). The BDNF-TrkB complex is endocytosed into a specialized vesicle, called a signaling endosome, which can induce a number of downstream signaling cascades locally, in the dendrites or distal axon. Although primarily, the BDNF-TrkB signaling endosome is trafficked to the cell soma via retrograde transport, where it initiates signaling pathways and causes changes in gene expression (Ginty and Segal, 2002; Cohen et al., 2011). Retrograde axonal transport is the mechanism by which signaling endosomes or cellular components packaged into vesicles are carried from the distal terminals of neurons to the cell body by the motor protein dynein (Maday et al., 2014). Disruption of this process is a hallmark of several neuromuscular diseases including spinal and bulbar muscular atrophy (SBMA),...
Huntington’s disease (HD), and amyotropic lateral sclerosis (ALS; Morfini et al., 2009). Because of the importance of BDNF-TrkB signaling for neuronal health and the initiation of retrograde transport, mice with reduced or absent muscle-synthesized BDNF were hypothesized to exhibit reduced TrkB activation at the neuromuscular junction (NMJ) as well as impairments in retrograde axonal transport (Mitchell et al., 2012). The Cre/lox gene technology system was used to generate experimental mice missing skeletal muscle-synthesized BDNF. Gastrocnemius (gastroc) muscle tissue was harvested from 120d animals, and the expression of TrkB and p-TrkB were assessed in the pre- and postsynaptic terminals of NMJs. Presynaptic TrkB expression was not statistically different between groups, while postsynaptic TrkB was significantly reduced in the heterozygous (HE) and homozygous (HO) knockout animals compared to controls. Pre- and postsynaptic p-TrkB expression was significantly reduced in both knockout mice compared to controls. These results suggest significantly decreased TrkB activation in gastroc-associated motor neurons of muscle-derived BDNF knockout animals. Sciatic nerve ligation experiments were performed on 120d mice to assess axonal transport mechanisms. Expression of the structural protein α-tubulin was evaluated as an internal control, and accumulation of TrkB and p-TrkB, along with c-Jun-amino-terminal kinase-interacting protein 3 (JIP3) and dynactin (DCTN1), two essential transport complex adaptor proteins, were assessed on the proximal and distal sides of ligation. Uniform levels of α-tubulin expression across groups indicated a high level of consistency throughout the immunohistochemical (IHC) staining and data collection procedures. On the proximal side of ligation, TrkB and DCTN1 accumulation were equivalent between HE knockout and control mice, while accumulation of p-TrkB and JIP3 was significantly
reduced in HE animals compared to controls, suggesting potential dysfunctions in anterograde axonal transport in HE knockouts. Distally, p-TrkB, JIP3, and DCTN1 accumulation were significantly reduced in HE mice compared to controls, indicating markedly impaired retrograde transport mechanisms. In HO knockout animals, accumulation of TrkB, p-TrkB, JIP3, and DCTN1, both proximally and distally, were significantly decreased when compared to control mice. These data suggest severe impairments in anterograde and retrograde axonal transport processes due to a lack of muscle-synthesized BDNF. Additionally, because of equivalent accumulation in HE and HO knockouts, the proper functioning of JIP3 was noted to exhibit some type of threshold requirement for the levels of muscle-BDNF expression.
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# TABLE OF CONTENTS

LIST OF FIGURES.......................................................................................................................... viii

LIST OF TABLES............................................................................................................................... xii

LIST OF ABBREVIATIONS.................................................................................................................... xiii

CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW........................................... 1

CHAPTER TWO: GENERATION OF EXPERIMENTAL TRANSGENIC ANIMALS.................................. 39
  Introduction......................................................................................................................................... 39
  Methods............................................................................................................................................... 41
    Animal husbandry............................................................................................................................ 41
    Genotyping of experimental animals............................................................................................... 42
    Breeding of heterozygous knockout animals.................................................................................... 44
    Breeding of homozygous knockout and control animals................................................................. 45

CHAPTER THREE: ASSESSMENT OF TRKB AND P-TRKB EXPRESSION IN GASTROC-ASSOCIATED NMJS OF MICE MISSING MUSCLE-SYNTHESIZED BDNF......................................................... 47
  Introduction......................................................................................................................................... 47
  Methods............................................................................................................................................... 48
    Animals............................................................................................................................................. 48
    Tissue harvest and processing.......................................................................................................... 49
    Alternate method of tissue harvest and processing........................................................................ 49
    TrkB immunohistochemistry............................................................................................................ 50
    p-TrkB immunohistochemistry.......................................................................................................... 51
    Imaging, data collection, and analysis.............................................................................................. 52
  Results................................................................................................................................................. 55
    TrkB results..................................................................................................................................... 55
    p-TrkB results................................................................................................................................. 57
  Discussion.......................................................................................................................................... 59

CHAPTER FOUR: EVALUATION AND CHARACTERIZATION OF RETROGRADE TRANSPORT IN MICE MISSING MUSCLE-SYNTHESIZED BDNF USING SCIATIC NERVE LIGATIONS.................................................. 63
  Introduction......................................................................................................................................... 63
Methods..........................................................................................................................65
  Animals....................................................................................................................65
  Sciatic nerve ligations and tissue harvest and processing..............................66
  TrkB immunohistochemistry............................................................................67
  p-TrkB immunohistochemistry.....................................................................68
  Imaging, data collection, and analysis.........................................................69
Results................................................................................................................................70
  TrkB results........................................................................................................70
  p-TrkB results..................................................................................................78
Discussion...............................................................................................................85

CHAPTER FIVE: SUMMARY AND CONCLUSIONS.................................................97

REFERENCES..........................................................................................................103

APPENDIX A: APPROVAL OF ANIMAL USE BY INSTITUTIONAL ANIMAL
  CARE AND USE COMMITTEE...........................................................................112
LIST OF FIGURES

Figure 1: Gastrocnemius myopathy in 120d transgenic mice missing muscle-synthesized BDNF .......................................................... 12

Figure 2: Ontogeny of progressive gastrocnemius myopathy in transgenic mice missing muscle-synthesized BDNF .......................................................... 13

Figure 3: Soleus myopathy in 120d transgenic mice missing muscle-synthesized BDNF .......................................................... 13

Figure 4: Ontogeny of soleus myopathy in transgenic mice missing muscle-synthesized BDNF .......................................................... 14

Figure 5: Mean presynaptic VAChT and postsynaptic AChR immunofluorescence in gastroc-associated NMJs of 120d transgenic mice missing muscle-synthesized BDNF .................................................................................. 15

Figure 6: Representative rendering of NMJ fragmentation in mice missing muscle-synthesized BDNF .................................................................................. 16

Figure 7: Multiple sample predictor model for gastroc NMJ fragmentation in mice missing muscle-synthesized BDNF .................................................................................. 16

Figure 8: Mean BDNF immunofluorescence in lumbar motor neurons of 30d and 120d mice missing muscle-synthesized BDNF .................................................................................. 18

Figure 9: Mean lamina X motor neuron cell soma area in 30d and 120d mice missing muscle-synthesized BDNF .................................................................................. 19

Figure 10: Mean dendritic diameter of Fluoro-Gold™ labeled gastroc-associated lumbar motor neurons in 30d and 120d mice missing muscle-synthesized BDNF .................................................................................. 19

Figure 11: Mean dendritic length of Fluoro-Gold™ labeled gastroc-associated lumbar motor neurons in 30d and 120d mice missing muscle-synthesized BDNF .................................................................................. 20

Figure 12: Cytoplasmic dynein and kinesin power axonal transport. Schematic diagram of the microtubule motor proteins cytoplasmic dynein and kinesin .................................................................................. 23

Figure 13: The ‘signaling endosome’ model .................................................................................. 25

Figure 14: Mean NF-H-P immunofluorescence at distal sciatic nerve ligation site in 30d and 120d mice missing muscle-synthesized BDNF .................................................................................. 31
Figure 15: Mean dynactin immunofluorescence at distal sciatic nerve ligation site in 120d mice missing muscle-synthesized BDNF...

Figure 16: Mean SYN immunofluorescence at distal sciatic nerve ligation site in 30d and 120d mice missing muscle-synthesized BDNF...

Figure 17: Mean NF-H-P immunofluorescence at proximal sciatic nerve ligation site in 120d mice missing muscle-synthesized BDNF...

Figure 18: Mean dynactin immunofluorescence at proximal sciatic nerve ligation site in 120d mice missing muscle-synthesized BDNF...

Figure 19: Mean SYN immunofluorescence at proximal sciatic nerve ligation site in 120d mice missing muscle-synthesized BDNF...

Figure 20: Mean NF-H-P immunofluorescence in axons and presynaptic terminals of gastroc-associated motor neurons in 30d and 120d mice missing muscle-synthesized BDNF...

Figure 21: Diagram detailing the tissue-specific targeting of BDNF in skeletal muscle using the Cre/lox system...

Figure 22: Results of BDNF gene PCR assay confirming loss of BDNF in skeletal muscle using the Cre/lox system...

Figure 23: Photograph of typical BDNF gene PCR amplification results on agarose gel...

Figure 24: Photograph of typical Cre transgene PCR amplification products on agarose gel...

Figure 25: Diagram of breeding scheme used to generate experimental transgenic animals...

Figure 26: Assessment of TrkB expression in gastroc-associated NMJs of 120d transgenic mice...

Figure 27: Evaluation of p-TrkB expression in gastroc-associated NMJs of 120d mice missing muscle-synthesized BDNF...

Figure 28: Assessment of TrkB immunofluorescence associated with VACHT-labeled presynaptic NMJs in 120d transgenic mice...

Figure 29: Evaluation of TrkB immunofluorescence associated with α-BTX-labeled postsynaptic NMJs in 120d transgenic mice...
Figure 30: Assessment of p-TrkB immunofluorescence associated with VACHT-labeled presynaptic NMJs in 120d transgenic mice.................................................................58

Figure 31: Evaluation of p-TrkB immunofluorescence associated with α-BTX-labeled postsynaptic NMJs in 120d transgenic mice.................................................................59

Figure 32: Assessment of protein accumulation on proximal side of ligated sciatic nerve in 120d MuscleBDNF+/− mouse..................................................................................70

Figure 33: Evaluation of α-tubulin immunofluorescence accumulation on proximal and distal portions of ligated sciatic nerve in 120d transgenic mice........................................71

Figure 34: Evaluation of TrkB immunofluorescence accumulation on proximal side of ligated sciatic nerve in 120d transgenic mice.................................................................73

Figure 35: TrkB immunofluorescence accumulation in distal portion of ligated sciatic nerve in 120d transgenic mice.....................................................................................73

Figure 36: Assessment of JIP3 immunofluorescence accumulation on proximal side of ligated sciatic nerve in 120d transgenic mice.................................................................75

Figure 37: JIP3 immunofluorescence accumulation in distal portion of ligated sciatic nerve in 120d transgenic mice.....................................................................................75

Figure 38: Assessment of DCTN1 immunofluorescence accumulation on proximal side of ligated sciatic nerve in 120d transgenic mice.............................................................77

Figure 39: DCTN1 immunofluorescence accumulation in distal portion of ligated sciatic nerve in 120d transgenic mice.....................................................................................77

Figure 40: Analysis of α-tubulin immunofluorescence accumulation on proximal and distal portions of ligated sciatic nerve in 120d transgenic mice........................................77

Figure 41: Evaluation of p-TrkB immunofluorescence accumulation on proximal side of ligated sciatic nerve in 120d transgenic mice.............................................................80

Figure 42: p-TrkB immunofluorescence accumulation in distal portion of ligated sciatic nerve in 120d transgenic mice.....................................................................................81

Figure 43: Assessment of JIP3 immunofluorescence accumulation on proximal side of ligated sciatic nerve in 120d transgenic mice.................................................................82

Figure 44: JIP3 immunofluorescence accumulation in distal portion of ligated sciatic nerve in 120d transgenic mice.....................................................................................83
Figure 45: Assessment of DCTN1 immunofluorescence accumulation on proximal side of ligated sciatic nerve in 120d transgenic mice................................................................. 84

Figure 46: DCTN1 immunofluorescence accumulation in distal portion of ligated sciatic nerve in 120d transgenic mice.......................................................................................... 85

Figure 47: Summary of sciatic nerve ligation protein accumulation results for TrkB IHC experiment.................................................................................................................. 92

Figure 48: Summary of sciatic nerve ligation protein accumulation results for p-TrkB IHC experiment.............................................................................................................. 93
LIST OF TABLES

Table 1: Gastrocnemius TrkB IHC secondary antibody labeling schemes.......................... 51
Table 2: Gastroc p-TrkB IHC secondary antibody labeling schemes.................................52
Table 3: Sciatic nerve ligation TrkB IHC secondary antibody labeling schemes.................. 68
Table 4: Sciatic nerve ligation p-TrkB IHC secondary antibody labeling schemes..............69
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AChR</td>
<td>acetylcholine receptor</td>
</tr>
<tr>
<td>ALS</td>
<td>amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>BC</td>
<td>bulbocavernosus</td>
</tr>
<tr>
<td>BCL2</td>
<td>B-cell leukemia/lymphoma 2</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CREB</td>
<td>cyclic AMP responsive element-binding protein</td>
</tr>
<tr>
<td>d</td>
<td>day(s)</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DCTN1</td>
<td>dynactin 1</td>
</tr>
<tr>
<td>df</td>
<td>degrees of freedom</td>
</tr>
<tr>
<td>DYNC1H1</td>
<td>cytoplasmic dynein 1 heavy chain 1</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase (also known as mitogen-activated protein kinase)</td>
</tr>
<tr>
<td>fALS</td>
<td>familial amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>floxed</td>
<td>loxP-flanked</td>
</tr>
<tr>
<td>Gastroc</td>
<td>gastrocnemius</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Haemotoxylin and Eosin</td>
</tr>
<tr>
<td>HD</td>
<td>Huntington’s disease</td>
</tr>
<tr>
<td>HE</td>
<td>heterozygous</td>
</tr>
<tr>
<td>HO</td>
<td>homozygous</td>
</tr>
<tr>
<td>HTT</td>
<td>huntingtin</td>
</tr>
<tr>
<td>IACUC</td>
<td>Institutional Animal Care and Use Committee</td>
</tr>
<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
</tr>
<tr>
<td>IL2</td>
<td>interleukin 2</td>
</tr>
<tr>
<td>IP</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>JIP3</td>
<td>c-Jun-amino-terminal kinase-interacting protein 3</td>
</tr>
<tr>
<td>LA</td>
<td>levator ani</td>
</tr>
<tr>
<td>NF-H</td>
<td>neurofilament-H</td>
</tr>
<tr>
<td>NF-H-P</td>
<td>phosphorylated neurofilament-H</td>
</tr>
<tr>
<td>NGF</td>
<td>nerve growth factor</td>
</tr>
<tr>
<td>NIH</td>
<td>National Institutes of Health</td>
</tr>
<tr>
<td>NMJ</td>
<td>neuromuscular junction</td>
</tr>
<tr>
<td>NT-3</td>
<td>neurotrophin-3</td>
</tr>
<tr>
<td>NT-4/5</td>
<td>neurotrophin-4 (also known as neurotrophin-5)</td>
</tr>
<tr>
<td>p75NTR</td>
<td>p75 neurotrophin receptor</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PI3-K</td>
<td>phosphatidylinositol 3-kinase</td>
</tr>
</tbody>
</table>
PLC-γ1 – phospholipase C gamma 1
polyQ – polyglutamine
p-Trk – phosphorylated-tropomyosin-related kinase receptor
sALS – sporadic amyotrophic lateral sclerosis
SBMA – spinal and bulbar muscular atrophy
SCT – spinal cord transection
SMA – spinal muscular atrophy
SNB – spinal nucleus of the bulbocavernosus
SYN – synaptophysin
Trk – tyrosine receptor kinase (also known as tropomyosin-related kinase)
VACHT – vesicular acetylcholine transporter
WT – wild type
α-BTX – α-bungarotoxin
CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

Neurotrophins and neurotrophin signaling

Neurotrophins are a family of small, diffusible proteins that strongly influence and regulate the development, survival, function, and plasticity of neurons. In the mammalian nervous system, neurotrophins are derived from a common ancestral gene, and thus are similar in sequence and structure (Hallböök, 1999; Huang and Reichardt, 2001). The known neurotrophins are nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5; Hallböök, 1999; Kaplan and Miller, 2000; reviewed in Huang and Reichardt, 2001; Reichardt, 2006). In addition to sequence and structural homologies, the individual neurotrophin genes also contain multiple promoters. The protein product of each gene is comprised of a signal sequence and a prodomain, followed by the mature neurotrophin sequence. The gene products, called proneurotrophins, must undergo proteolytic cleavage to form the mature neurotrophin protein. Unsurprisingly, this is an important post-transcriptional point of regulation that both limits and adds to the specificity of neurotrophin action (Lee et al., 2001b; Reichardt, 2006). Functions of the neurotrophin prodomains include promoting the folding of the mature domain and sorting of neurotrophins to either constitutive or regulated secretory pathways. Furthermore, the highly conserved nature of the prodomains suggest they may mediate additional biological actions and have an important role in intracellular processing (Heinrich and Lum, 2000; reviewed in Lu et al., 2005).
Neurotrophins exert their actions by binding to two types of membrane receptors – the high-affinity tyrosine receptor kinase or tropomyosin receptor kinase (Trk) family of receptors, and the lower-affinity p75 neurotrophin receptor (p75NTR), a member of the tumor necrosis factor receptor superfamily (McAllister et al., 1999; Kaplan and Miller, 2000; Patapoutian and Reichardt, 2001). Past work has shown that proneurotrophins primarily bind p75NTR, while the mature neurotrophins bind to the Trk receptors (Lu et al., 2005). The Trk receptor isoforms have a degree of specificity as to the particular neurotrophin they will bind. For example, NGF has a high affinity for the tropomyosin-related kinase A (TrkA) receptor. BDNF and NT-4/5 bind to the TrkB receptor. NT-3, on the other hand, primarily activates the TrkC receptor, but is also recognized by TrkA and TrkB receptors, although with less affinity than the primary ligands (Huang and Reichardt, 2001; Patapoutian and Reichardt, 2001). Several studies have shown that p75NTR can increase both the affinity and specificity of Trk receptor-neurotrophin interactions and induce apoptosis in neurons and other cell types when Trk activation is reduced or absent (Lee et al., 2001a, 2001b). The p75NTR and Trk receptors orchestrate and modify neuronal responses to neurotrophins and are often located on the same cell. Trk receptors, however, generally transmit signals that lead to neural growth and synaptic expansion, while p75NTR signaling often leads to cellular hypotrophy and apoptosis (reviewed in Kaplan and Miller, 2000; Lu et al., 2005). The pro-survival effects of mature neurotrophins have been well established (reviewed in Lu et al., 2005). Administration of mature neurotrophins to specific brain regions can rescue neuronal loss associated with aging or induced by chemical or mechanical insults, while deletion of specific neurotrophin genes leads to the loss of certain populations of neurons (reviewed...
Additionally, neurotrophins are important in both developing and mature neural circuits. These studies are discussed in more detail below.

There are many stages involved in the development of neural circuits, and neurotrophins have critical roles in each. The stages of circuit development include neural stem cell survival and differentiation, axon-dendrite differentiation, axonal growth and guidance, synapse formation and maturation, and refinement of developing circuits (reviewed in Park and Poo, 2012). Embryonic neural stem cells express Trk receptors and p75NTR (Park and Poo, 2012). Treating cultured cortical and hippocampal neural stem cells from mouse embryos with recombinant BDNF and NGF promotes cell survival and differentiation into neurons (Ahmed et al., 1995; Lachyankar et al., 1997; Shetty and Turner, 1998; reviewed in Park and Poo, 2012). During axon-dendrite polarization of cultured hippocampal neurons, exposure of an undifferentiated neurite to extracellular BDNF promotes its differentiation into an axon (Shelly et al., 2007). In cultured neurons, an extracellular gradient of neurotrophins causes chemotropic turning of axonal growth cones (Gundersen and Barrett, 1979; Song et al., 1997). This guidance may also occur in vivo. In developing mouse limb buds, sensory axons can be directed toward implanted beads containing NGF, BDNF, NT-3, or NT-4/5 (Tucker et al., 2001). In mature pyramidal cells in developing cortical slices, dendritic growth is increased by treatment with BDNF, but inhibited by NT-3 in layer 4. In layer 6, these effects are reversed (McAllister et al., 1997). In the developing optic tectum of Xenopus laevis tadpoles, in vivo infusion of exogenous BDNF promotes synaptogenesis; this phenomenon may be attributed to the BDNF-induced increases in axon and dendrite arborization of retinal ganglion cells (Cohen-Cory and Fraser, 1995; McAllister et al., 1995, 1996; Alsina et al.,
The actions of neurotrophins in the development of neural circuits are reviewed in further detail by Park and Poo, 2012.

In addition to the numerous roles neurotrophins play in neural development, these proteins also serve many regulatory functions in mature circuits (reviewed in Park and Poo, 2012). Neurotrophins released at the synapse can rapidly change the efficacy of synaptic transmission and the capacity of activity induced by long-term potentiation and long-term depression (reviewed in Park and Poo, 2012). Neurotrophins mediate changes in neuronal excitability through their effects on the expression and function of voltage-gated ion channels (reviewed in Park and Poo, 2012).

Neurotrophin-mediated activation of Trk receptors regulate numerous pathways such as proliferation and survival of neurons, axonal and dendritic growth, assembly and remodeling of the cytoskeleton, membrane trafficking and fusion, and synapse formation and plasticity (reviewed in Huang and Reichardt, 2003). The importance of Trk receptor activation is underscored by the loss of specific cell populations in Trk gene knockouts (reviewed in Lu et al., 2005). TrkB-deficient mice show reduced survival of newly generated hippocampal granule cells (Sairanen, 2005; Bergami et al., 2008). Additionally, mice lacking TrkB in hippocampal neural progenitor cells have impaired proliferation and neurogenesis (Bath et al., 2008; Li et al., 2008). Ligand activation of Trk receptors results in autophosphorylation of tyrosine residues on the cytoplasmic domain, leading to the activated form – phosphorylated-Trk (p-Trk). Of the 10 evolutionarily conserved tyrosines in their cytoplasmic domains, three are present in the autoregulatory loop of the kinase domain that mediate tyrosine kinase activity. Phosphorylation of these residues further enhances receptor activation (reviewed in

2001).
Huang and Reichardt, 2001). Trk receptor signaling is promoted by phosphorylation of the other tyrosine residues, which creates docking sites for adapter proteins containing phosphotyrosine-binding or src-homology-2 motifs (reviewed in Huang and Reichardt, 2001). The main pathways induced by Trk receptor activation include the Ras, Rac, and phosphatidylinositol 3-kinase (PI3-K) cascades, the phospholipase C-γ1 (PLC-γ1) pathway, as well as their downstream effectors (reviewed in Huang and Reichardt, 2003; Reichardt, 2006). PI3-K activation of protein kinase B suppresses cell death by inhibiting the apoptotic activities of forkhead and B-cell leukemia/lymphoma 2 (BCL2)-associated death protein (Datta et al., 1997; del Peso et al., 1997; Brunet et al., 1999; reviewed in Lu et al., 2005; Reichardt, 2006). Ras stimulation of mitogen-activated protein kinase (also known as extracellular signal-regulated kinase, ERK) signaling cascades produces a number of downstream effects, including increased activity or expression of anti-apoptotic proteins such as BCL2 and the transcription factor cyclic AMP responsive element-binding protein (CREB; Aloyz et al., 1998; Riccio et al., 1999; reviewed in Lu et al., 2005; Reichardt, 2006). PLC-γ1-dependent generation of inositol triphosphate and diacylglycerol (DAG) leads to mobilization of Ca\(^{2+}\) stores and activation of Ca\(^{2+}\)- and DAG-regulated protein kinases, including protein kinase C. Studies have shown that the endocytosis and transfer of Trk receptors to different membrane compartments, in conjunction with the localization of many adapter proteins to specific membrane compartments, modulates the efficiency and duration of Trk-mediated signaling (Geetha et al., 2005; reviewed in Reichardt, 2006).
Importance of brain-derived neurotrophic factor (BDNF)

BDNF is the most widely expressed and well-characterized neurotrophin in the mammalian brain (Anastasia and Hempstead, 2014). Despite its name, BDNF is synthesized in a number of other tissue types besides the brain, including skeletal muscle, endocrine tissues, and male- and female-specific tissues such as the testis and fallopian tubes (Funakoshi et al., 1995; Matthews et al., 2009; Halievski et al., 2015). Studies have shown that proBDNF is the main form secreted from cells, and mature BDNF is generated via proteolytic cleavage by extracellular proteases. BDNF, proBDNF, and the cleaved prodomain are all biologically active (Lu et al., 2005; Anastasia and Hempstead, 2014). In addition to the pro-survival effects of BDNF signaling, BDNF has been shown to rapidly modulate the synthesis, metabolism, and release of neurotransmitters such as glutamate (reviewed in Park and Poo, 2012). Moreover, BDNF-TrkB signaling regulates the organization of the cytoskeleton, influences cell motility and growth cone behavior, and modulates synaptic function and plasticity (reviewed in Reichardt, 2006; Park and Poo, 2012).

BDNF acts as an anterograde signal protein, binding to post-synaptic receptors in both the central nervous system (CNS) and peripheral nervous system (Zhou and Rush, 1996; Altar et al., 1997; Conner et al., 1997). Additionally, BDNF promotes axon development and growth in both developing and polarized neurons in an autocrine fashion by acting on the cell that secreted it (Cheng et al., 2011). BDNF also acts as a retrograde signal protein and can be secreted from target (postsynaptic) tissue to subsequently bind to presynaptic receptors (Ginty and Segal, 2002). After binding and activating its presynaptic receptor, often the BDNF-TrkB complex is endocytosed and
then transported to the cell body in retrograde signaling endosomes by the cytoskeletal motor protein dynein (Ginty and Segal, 2002; Howe and Mobley, 2005; Cosker and Segal, 2014; Ayloo et al., 2017). At the cell body, the BDNF-TrkB complex activates downstream signaling cascades and can induce changes in gene expression (Ginty and Segal, 2002). Thus, BDNF activation of TrkB can initiate signaling cascades (mentioned previously) at a postsynaptic cell, at the presynapse, and in the cellular perikarya (Ginty and Segal, 2002).

Irregularities in the levels and activities of a variety of neurotrophins have been implicated in a number of neurological disorders. BDNF is understood as one of the major regulators of synaptic plasticity, neuronal survival and differentiation, and as such, is a leading target for drug therapies (Zuccato and Cattaneo, 2009). In the early 2000s, a single-nucleotide polymorphism in the BDNF gene that leads to a valine → methionine substitution at position 66 in the prodomain (Val66Met; BDNF<sub>Met</sub>) was linked to memory impairments, reductions in hippocampal volume, and altered susceptibility to disorders such as Alzheimer’s disease, Parkinson’s disease, depression, eating disorders, and bipolar disorder (Chao et al., 2006; Anastasia and Hempstead, 2014). The Val66Met substitution impairs BDNF release from neurons by altering the structure of its prodomain, which, in a p75NTR-dependent manner, ultimately decreases Rac signaling leading to neuronal growth cone reduction and a loss in synaptic density (Anastasia and Hempstead, 2014).

A number of studies performed in postmortem humans have demonstrated that BDNF mRNA expression is reduced in the substantia nigra of Parkinson’s disease patients and in affected cortical regions in Alzheimer’s disease (Holsinger et al., 2000;
Howells et al., 2000; Liu et al., 2005). While homozygous universal knockout of the
*BDNF* gene in mice leads to embryonic death and rare chances of survival, heterozygous
*BDNF* knockouts display enhanced inter-male aggressiveness, hyperphagia, and
significant weight gains in early adulthood (Ernfors et al., 1994; Lyons et al., 1999). In
addition, a reduction in BDNF mRNA is a hallmark of several neurodegenerative
diseases including amyotrophic lateral sclerosis (ALS), spinal muscular atrophy (SMA),
spinal and bulbar muscular atrophy (SBMA), Huntington’s disease (HD), Alzheimer’s
disease, and Parkinson’s disease (Holsinger et al., 2000; Howells et al., 2000; Zuccato et
al., 2001; Jiang et al., 2005; Liu et al., 2005; Zuccato and Cattaneo, 2009; Halievski et al.,
2015).

While the role of BDNF in the neurons and glia of the brain and spinal cord has
been studied extensively, its actions in other tissue types are less understood. In the late
1990s and early 2000s, researchers noted that physical activity preserved cognitive
function in elderly populations, promoted functional recovery after traumatic CNS injury,
and induced neurogenesis in the adult CNS, likely by increasing neurotrophin production
in select regions of the brain and spinal cord (Neeper et al., 1995; Jones et al., 1999;
Researchers found that application of BDNF to severed spinal cord promoted
regenerative motor neuron growth and stimulated hindlimb stepping (Bregman et al.,
1997; Jakeman et al., 1998; Yang and Arnold, 2000; Gomez-Pinilla et al., 2002; Yang et
al., 2004). Gómez-Pinilla et al. (2002) demonstrated that voluntary exercise increased
BDNF and CREB mRNA expression in spinal cord and soleus muscle of rats. In the
spinal cord, CREB mRNA expression increased in proportion to BDNF mRNA levels
(Gomez-Pinilla et al., 2002). In the same study, researchers noted that paralysis of the soleus muscle using botulinum toxin type A reduced BDNF mRNA levels below control levels and prevented any increases in spinal cord and muscle ensuing from physical activity (Gomez-Pinilla et al., 2002). The results obtained by Gómez-Pinilla et al. (2002) suggest that basal levels of neuromuscular activity are required to maintain normal levels of BDNF in the neuromuscular system and ultimately, the potential for neuroplasticity.

Another study emphasizing the existence of a signaling pathway between physical activity and neuronal changes was conducted by Gazula and colleagues in 2004. In this experiment, the dendritic structure of motor neurons of rats with an intact spinal cord was compared to rats with a complete spinal cord transection (SCT) and rats with a SCT that were also engaged in daily exercise consisting of actively moving paralyzed hindlimbs through the motions of walking. The motor neurons of animals with SCT showed considerable atrophy, displaying loss of dendritic membrane and branching when compared to rats with intact spinal cords (Gazula et al., 2004). In contrast, none of these detrimental changes were observed in motor neurons of rats with SCT who underwent the subsequent exercise regime (Gazula et al., 2004). In a second experiment by the same group, researchers noted that increased exercise in rats with intact spinal cords had minimal effects on dendritic morphology, suggesting an upper limit of activity-dependent dendritic growth (Gazula et al., 2004). The results obtained by these experiments, along with those of Gómez-Pinilla et al., suggest the influence of muscle-derived signaling in the maintenance of motor neurons as well as recovery following injury (Gomez-Pinilla et al., 2001, 2002; Gazula et al., 2004).
The importance of muscle-synthesized BDNF signaling in the health and maintenance of innervating neurons is also highlighted by studies investigating the spinal nucleus of the bulbocavernosus (SNB). This sexually dimorphic group of motor neurons is found near the midline of the ventral horn in the lower lumbar spinal cord of rats. In males, SNB motor neurons innervate the bulbocavernosus (BC) and levator ani (LA) muscles which attach to the penis and control penile reflexes and copulation (Breedlove and Arnold, 1980; Cooke et al., 1998; Ottem et al., 2007). The SNB motor neurons in males have a high degree of dendritic arborization, and their development and maintenance are dependent on androgens as is evidenced by dendritic retraction following castration (Kurz et al., 1986; Goldstein et al., 1990; Ottem et al., 2007). Following castration, androgen replacement protocols restore the dendritic arborization (Kurz et al., 1986; Goldstein et al., 1990; Ottem et al., 2007). Additionally, axotomy of adult SNB motor neurons decreases their levels of BDNF, which suggests that BC and LA muscles are a source of BDNF for these cells (Yang and Arnold, 2000). Axotomy results in significant size reductions of SNB somata, while BDNF treatment at the site of the severed axons counteracts this effect (Yang and Arnold, 2000). In these androgen-sensitive neurons, BDNF treatment alone does not restore the dendrites of SNB motor neurons, and requires the combinatory action of testosterone replacement to achieve dendritic restoration (Yang et al., 2004). An early study emphasizing the role of BDNF signaling from BC and LA muscles in the modulation of SNB motor neurons showed that disruption of retrograde signal transport, not lack of muscle activity, was responsible for reductions in androgen receptor expression in SNB motor neurons (reviewed in Ottem et al., 2013).
Importance of muscle-synthesized BDNF

Building on these studies, the Ottem research laboratory has employed the use of a transgenic mouse model to further the understanding of the role of muscle-synthesized BDNF. By utilizing the Cre/lox gene technology system (described in more detail in Chapter 2), mice with a tissue-specific loss, or knockout, of one or both copies of the BDNF gene in skeletal muscle were generated and used for experiments. These mice display a behavioral phenotype that is similar to that observed in other animal models of neuromuscular diseases such as ALS or SBMA (Moser et al., 2013). For example, the adult heterozygous (Muscle^{BDNF+/−}) and homozygous (Muscle^{BDNF−/−}) knockout mice struggle to remain on a suspended rotating rod, have reduced grip strength, and have higher clasping scores compared to wild type control animals (WT; Muscle^{BDNF+/+}). In addition, histological analyses revealed significant muscle pathology in the gastrocnemius (gastroc) muscle of heterozygous and homozygous knockout mice. These mice exhibit hypertrophic and hypotrophic fibers, fiber splitting, centralized nuclei, and an overall age-independent, but progressive loss of fibers that was apparent when comparing muscles from 30d, 90d, 120d, 180d, and 210d animals (Figures 1 and 2). The myopathology is not as pervasive in other muscles examined, which may be attributed to the varying composition of fiber types in each muscle. For example, in the slow-twitch muscle of the soleus, 30d homozygous knockouts were the only group to display increased myopathology when compared to control animals at any older age (Figures 3 and 4).
Figure 1. Gastrocnemius (gastroc) muscle fiber pathology in 120d transgenic mice missing muscle-synthesized BDNF. Gastroc muscle sections (40 µm) were stained with Haemotoxylin and Eosin (H&E) and photomicrographs were taken at 10X. (A) Wild type control (WT; Muscle$^{BDNF+/+}$) fibers displayed no splitting and nuclei were localized in the periphery of the fibers. (B) Heterozygous knockout (Muscle$^{BDNF+/−}$) muscle fibers showed fiber splitting (triangle) and centralized nuclei (arrows). (C) In addition to fiber splitting (triangles) and centralized nuclei (arrows), homozygous knockout (Muscle$^{BDNF−/−}$) muscle tissue exhibited considerable hyper- and hypotrophy. Adapted from Taisto et al. (2013).
Figure 2. Ontogeny of gastrocnemius (gastroc) myopathology in Muscle\(^{BDNF+/+}\), Muscle\(^{BDNF+/-}\), and Muscle\(^{BDNF-/-}\) mice. In addition to the significant increases in mean percentage of gastroc fibers displaying myopathy in heterozygous and homozygous knockouts, there was an age-independent, but progressive escalation of myopathy in these animals (*** = p<0.001). Adapted from Taisto et al. (2013).

Figure 3. Soleus myopathology in 120d transgenic mice missing muscle-synthesized BDNF. Muscle sections (40 µm) were H&E stained and photomicrographs were taken at 10X. (A) Wild type control (WT; Muscle\(^{BDNF+/+}\)) fibers displayed no splitting and nuclei were localized in the periphery of the fibers. (B) Heterozygous (Muscle\(^{BDNF+/-}\)) and (C) homozygous (Muscle\(^{BDNF-/-}\)) knockout muscle fibers exhibited some fiber splitting (triangle) and centralized nuclei (arrows). Adapted from Taisto et al. (2013).
Figure 4. Ontogeny of soleus myopathy in Muscle\textsuperscript{BDNF\,+\,+}, Muscle\textsuperscript{BDNF\,+/-}, and Muscle\textsuperscript{BDNF\,-/-} mice. Significant increases in mean percentage of soleus muscle fibers displaying myopathy were recorded only for 30d homozygous (Muscle\textsuperscript{BDNF\,-/-}) knockouts compared to control animals (** = p<0.001). Adapted from Taisto et al. (2013).

In addition to the myopathy recorded in transgenic mice missing muscle-synthesized BDNF, the morphology of gastroc and soleus motor neurons were also investigated using a series of histological and immunohistochemical (IHC) assays to determine soma size and dendritic arborization of the associated lumbar motor neurons. Utilizing IHC, proteins associated with the neuromuscular junction (NMJ) could be compared between WT animals and those with reduced or absent muscle-synthesized BDNF. By labeling vesicular acetylcholine transporter (VACHT; a presynaptic marker), and the postsynaptic acetylcholine receptor (AChR) using α-bungarotoxin (α-BTX; a postsynaptic marker), the presynapse and postsynapse of the NMJ were visualized and
then evaluated. Comparing immunofluorescence labeling of gastroc-associated NMJs between the experimental groups of 120d mice revealed a significant reduction in the presynaptic surface area in homozygous knockout (Muscle\textsuperscript{BDNF-/}) mice (Figure 5A), whereas there were no significant differences in the postsynaptic surface area between genotypes (Figure 5B). Fragmentation of the NMJ, which manifests naturally as a result of aging and/or dystrophy, was found to have a significantly higher occurrence in heterozygous and homozygous knockout animals compared to controls (Figures 6 and 7). These results may be due to impaired TrkB signaling, which has been shown to induce disassembly and fragmentation of postsynaptic AChR clusters (Gonzalez et al., 1999; Kulakowski et al., 2011).

![Figure 5](image_url)

**Figure 5.** Mean surface area (μm\(^2\)) of presynaptic vesicular acetylcholine transporter (VACHT) and postsynaptic acetylcholine receptor (AChR; labeled with α-BTX) immunofluorescence in gastroc-associated neuromuscular junctions of 120d animals. (A) There was a marked decrease in presynaptic VACHT surface area in homozygous (Muscle\textsuperscript{BDNF-/}) knockouts compared to controls (** p<0.001). (B) There were no significant differences in postsynaptic AChR surface area between animal groups. Adapted from Taisto et al. (2013).
Figure 6. Representative Imaris (Bitplane) rendering of neuromuscular junction (NMJ) fragmentation in mice missing muscle-synthesized BDNF. Presynaptic VACHT is shown in cyan, while postsynaptic AChR (labeled with α-BTX) is in red. (A) Control (Muscle$^{BDNF+/+}$) animals displayed complete, intact NMJs. (B) Heterozygous (Muscle$^{BDNF+/-}$) and (C) homozygous (Muscle$^{BDNF-/-}$) knockout mice exhibited genotype-dependent increases in NMJ fragmentation. Adapted from Taisto et al. (2013).

Figure 7. Multiple sample predictor model for gastroc NMJ fragmentation in mice missing muscle-synthesized BDNF. Probability of gastroc-associated NMJ fragmentation was significantly higher in heterozygous (Muscle$^{BDNF+/-}$) and homozygous (Muscle$^{BDNF-/-}$) knockout mice compared to controls (Muscle$^{BDNF+/+}$; ** = p<0.01; *** = p<0.001). Adapted from Taisto et al. (2013).
Cellular pathologies have been recorded in gastroc-associated motor neurons in transgenic mice missing muscle-synthesized BDNF (Pomeroy, 2013). Notably, in lumbar motor neurons of 30d mice, Muscle^{BDNF−/−} animals showed significantly increased BDNF immunofluorescence compared to control (Muscle^{BDNF+/+}) and heterozygous knockout mice (Muscle^{BDNF+/−}; Figure 8A). In contrast, 120d heterozygous animals exhibited a marked increase in BDNF immunofluorescence compared to the other groups (Figure 8B). Furthermore, mean motor neuron cell soma area was significantly diminished in heterozygous and homozygous knockouts compared to controls in both 30d and 120d animals (Figures 9A and 9B, respectively). In another experiment, the fluorescent tracer molecule Fluoro-Gold™ (Fluorochrome LLC, Denver, CO), also known as hydroxystilbamidine, was utilized to assess lumbar motor neuron morphology. Following injection into gastroc muscle, Fluoro-Gold™ is endocytosed by innervating motor neurons and carried back to the cell body via retrograde axonal transport, where it fills the soma and dendrites (Schmued and Fallon, 1986). Thus, mean Fluoro-Gold™-labeled dendritic diameter and length were able to be quantified and compared for experimental groups at 30d and 120d. As depicted in Figure 10, the mean dendritic diameter was significantly reduced in 30d homozygous knockout animals compared to other groups, while at 120d, both heterozygous and homozygous knockout animals had reduced dendritic diameter compared to controls (Pomeroy, 2013). The mean dendritic length of gastroc-associated lumbar motor neurons was significantly reduced in 30d heterozygous and homozygous knockout animals compared to controls (Figure 11; Pomeroy, 2013). At 120d, there were general increases in mean dendritic length for each genotype, but again, dendritic length was significantly diminished in Muscle^{BDNF+/−} and Muscle^{BDNF−/−} mice.
compared to control animals (Figure 11; Pomeroy, 2013). However, these reductions in dendritic length and diameter may have been due to impairments in retrograde axonal transport rather than abnormalities in motor neuron morphology, as discussed later (Pomeroy, 2013; Dangremond, 2016).

Figure 8. Mean BDNF immunofluorescence in lumbar motor neurons of 30d and 120d mice. (A) At 30d, lumbar motor neurons of homozygous (Muscle\textsuperscript{BDNF/\textminus}) knockouts exhibited a marked increase in somal BDNF immunofluorescence compared to controls, whereas no significant changes were observed in heterozygous (Muscle\textsuperscript{BDNF/+\textminus}) mice compared to control animals (*** = p<0.001). (B) In contrast, at 120d, somal BDNF immunofluorescence was significantly higher in Muscle\textsuperscript{BDNF/+\textminus} mice compared to controls, while there were no notable differences in Muscle\textsuperscript{BDNF/-} animals compared to controls (*** = p<0.001). Adapted from Pomeroy (2013).
Figure 9. Mean lamina X motor neuron cell soma area (µm²) in 30d and 120d Muscle\textsuperscript{BDNF+/+}, Muscle\textsuperscript{BDNF+/-}, and Muscle\textsuperscript{BDNF-/-} mice. (A) At 30d and (B) 120d, both heterozygous and homozygous knockouts displayed significant reductions in area compared to controls (** = p<0.01; *** = p<0.001). Adapted from Pomeroy (2013).

Figure 10. Mean dendritic diameter (µm) of Fluoro-Gold™ labeled gastroc-associated lumbar motor neurons in Muscle\textsuperscript{BDNF+/+}, Muscle\textsuperscript{BDNF+/-}, and Muscle\textsuperscript{BDNF-/-} mice. At 30d, only homozygous knockouts exhibited significant reductions in dendritic diameter compared to control mice (** = p<0.001). Whereas at 120d, both heterozygous and homozygous knockout animals displayed diminished dendritic diameter compared to controls (** = p<0.001). Adapted from Pomeroy (2013).
Figure 11. Mean dendritic length (µm) of Fluoro-Gold™ labeled gastro-associat
ed lumbar motor neurons in 30d and 120d Muscle$^{BDNF+/+}$, Muscle$^{BDNF+/-}$, and Muscle$^{BDNF-/-}$ mice. At 30d, both heterozygous and homozygous knockouts displayed decreased

dendritic length compared to controls (** *= p<0.001). By 120d, there was an overall

increase in dendritic length, however, hetero- and homozygous knockout mice still

exhibited significant reductions in dendritic length compared to controls (** *= p<0.001).

Adapted from Pomeroy (2013).

**Axonal transport mechanisms**

Disruption of retrograde axonal transport in motor neurons is a hallmark of

several neuromuscular diseases including SBMA, HD, and ALS (Morfini et al., 2009).

Retrograde transport is the process by which proteins, mitochondria, and other cellular

components are packaged into vesicles in the distal synaptic terminals of neurons, and

then retrogradely transported toward the cell body by the cytoskeletal motor protein

(cytoplasmic) dynein (Goldstein and Yang, 2000). Cytoplasmic dynein is a large and

complex molecule composed of two heavy chains (DYNC1H1), which fold to form the

motor domains and utilize ATP hydrolysis to generate force, as well as a number of
intermediate, light intermediate, and light chains, which are thought to function primarily in cargo association and regulation (Chevalier-Larsen and Holzbaur, 2006). A schematic of this complex is shown in Figure 12. In addition to cellular cargo, dynein is responsible for the retrograde movement of neurotrophin signaling endosomes, including BDNF, through the long axons of motor neurons (Cosker and Segal, 2014). Moreover, studies have shown that dynein is able to transport cargo and signaling endosomes both into and out of dendrites, further emphasizing the importance of this motor protein (Kapitein et al., 2010; Ayloo et al., 2017).

Recently, research has begun to more-closely examine the nuanced mechanics of long-range transit of cellular cargo by dynein and kinesin motors. One such study revealed that kinesin-1 moves cargo less efficiently in hippocampal dendrites compared to axons, suggesting a compartment-based specificity for this motor protein (Ayloo et al., 2017). Conversely, dynein was noted to effectively navigate both axons and dendrites (Ayloo et al., 2017). Additionally, dynein-dependent transport in axons and dendrites was increased by dynamic microtubule plus ends, which has been shown to be the result of an enhanced interaction of dynactin with tyrosinated microtubules (McKenney et al., 2016; Nirschl et al., 2016; Ayloo et al., 2017).

Dynactin, an accessory or activator complex, helps dynein bind cargo and signaling endosomes, has been demonstrated to be required for most dynein functions in vivo, and is important for the initiation of axonal transport (Chevalier-Larsen and Holzbaur, 2006; Moughamian and Holzbaur, 2012; Moughamian et al., 2013; Cosker and Segal, 2014; McKenney et al., 2014, 2016; Nirschl et al., 2016). Dynactin is a large, complex molecule composed of 11 different subunits (Chevalier-Larsen and Holzbaur,
2006). The most notable aspect of dynactin is the actin-like filament subunit, actin-related protein Arp1, which forms the base of the complex (Chevalier-Larsen and Holzbaur, 2006). Projecting from this filament is a dimer of an extended coil-coil protein, p150Glued, which binds directly to dynein and microtubules (Chevalier-Larsen and Holzbaur, 2006). A schematic of dynactin associated with the cytoplasmic dynein motor complex is shown below in Figure 12.
A number of studies have demonstrated the importance of neurotrophin signaling via Trk receptors for the initiation of retrograde transport. Yano et al. (2001) performed coimmunoprecipitation experiments in vivo to demonstrate that Trk receptors, including TrkB, can form a complex with the light and intermediate chains of dynein. In the same study, researchers employed sciatic nerve ligations to show marked accumulation of
TrkB with light and intermediate chains of dynein, suggesting a direct interaction between TrkB and the dynein complex (Yano et al., 2001). Dynein has been shown to be required for the proper retrograde transport of BDNF-TrkB complexes, however, other motors may also contribute to this motility (Ghiretti et al., 2016; Ayloo et al., 2017). Endosomes containing neurotrophin-Trk receptor complexes associate with the dynein motor complex, and this is known as the signaling endosome model (Figure 13). The major function of BDNF-TrkB signaling endosomes is to modulate gene expression following active transport to the soma (Heerssen et al., 2004; Cohen et al., 2011; Ayloo et al., 2017). The signaling endosome model is supported by a number of studies demonstrating that after formation, the neurotrophin-Trk complex is internalized into endosomes through a clathrin-mediated endocytotic pathway, and transported retrogradely toward the cell soma (Ginty and Segal, 2002; Heerssen et al., 2004; Maday et al., 2014; Ghiretti et al., 2016; Ayloo et al., 2017). Additional studies have shown that TrkB activation of the Erk1/2 kinase pathway recruits cytoplasmic dynein to signaling endosomes for retrograde axonal transport (Mitchell et al., 2012). Moreover, inhibition of Erk1/2 reduces the motility of TrkB-containing endosomes and the extent of their colocalization with dynein in axons (Mitchell et al., 2012). High-resolution imaging has also demonstrated dynein-based vesicular transport of activated Trk (p-Trk) receptors within rat sciatic nerve axons in a retrograde fashion, as vesicles accumulate distal to a ligation site (Bhattacharyya et al., 2002). One study utilized kinase-dead and truncated variants of TrkB to show that only activated receptors undergo retrograde transport (Heerssen et al., 2004).
Other studies have described the significance of adaptor proteins, such as c-Jun NH\textsubscript{2}-terminal kinase-interacting protein 3 (JIP3), in the regulation of Trk receptor axonal transport and augmentation of neurotrophin signaling by directly linking Trk with motor protein complexes (Huang et al., 2011). Additionally, the binding of JIP3 to kinesin-l light chain has been shown to be essential for anterograde TrkB transport and BDNF-induced retrograde TrkB signaling (Sun et al., 2017).

Figure 13. The ‘signaling endosome’ model. Trk receptors on distal axons are activated upon binding to neurotrophin. The ligand-receptor complex internalizes through clathrin-mediated endocytosis. Some of the vesicles become specialized endosomes that serve as platforms for continued Trk signaling and are transported retrogradely to the cell body using a dynein-dependent and microtubule-dependent transport mechanism. The vesicle-associated Trk receptor remains autophosphorylated and capable of promoting a unique set of signals upon arrival at the cell bodies. These include PI3K and Erk5. Adapted from Ginty and Segal (2002).

Axonal transport deficits in disease

Defects in axonal transport may explain the heightened vulnerability of long projection neurons, such as motor neurons, in diseases such as SBMA, HD, and ALS (Morfini et al., 2009; Kemp et al., 2011). The cell body of motor neurons is the primary site of both metabolic and degradative functions, and thus requires continuous, efficient
transport of materials to and from the cell soma (Chevalier-Larsen and Holzbaur, 2006; Duncan and Goldstein, 2006). Mutations that affect the cytoplasmic dynein motor complex, specifically the gene encoding DYNC1H1, have been shown to cause spinal muscular atrophy (SMA) and Charcot-Marie-Tooth disease (Garrett et al., 2014). One study in particular demonstrated that one such mutation, in a mouse model of SMA, altered signaling endosome transport kinetics as well as Erk1/2-cFos signaling in a cell-type specific manner, suggesting a plausible explanation for how mutations in ubiquitously expressed DYNC1H1 cause neuron-specific disease (Garrett et al., 2014).

This theme, ubiquitous expression of a causative gene resulting in selective neuronal death and dysfunction in disease-specific areas of the CNS, is common, and suggests similar molecular mechanisms for these diseases (Katsuno et al., 2006). For example, HD is caused by an abnormal polyglutamine (polyQ) expansion in huntingtin protein (HTT), and is characterized by the loss of striatal and cortical neurons (Liot et al., 2013). Similarly, spinal and bulbar muscular atrophy (SBMA, or Kennedy’s disease) is characterized by the loss of bulbar and spinal motor neurons, and is caused by an expansion of the polyQ tract in the first exon of the androgen receptor (AR) gene (Katsuno et al., 2006). Both of these diseases have been linked to disruptions in retrograde transport, as discussed below.

Studies have shown that vesicular transport deficits, particularly of BDNF, are a major aspect of the cellular pathology in HD, as BDNF is not produced by striatal neurons, but is synthesized in the cortex and transported to the striatum by corticostriatal projecting neurons (Altar et al., 1997; Liot et al., 2013). While reducing cortical production of BDNF leads to striatal degeneration, recovery of axonal transport or
secretion of BDNF enhances BDNF release and neuronal survival, suggesting that BDNF from cortical neurons activate TrkB receptors at striatal dendrites to promote striatum survival (Liot et al., 2013). In a set of experiments by Liot and colleagues, TrkB was demonstrated to bind and colocalize with HTT and dynein, silencing HTT reduced vesicular transport of TrkB in striatal neurons, and, in HD, the polyQ expansion in HTT altered the binding of TrkB-containing vesicles to microtubules and reduced transport, suggesting HTT as a critical mediator of TrkB trafficking (Liot et al., 2013).

Impaired retrograde transport has also been implicated in SBMA, where the mutant AR gene causes aggregation of the deviant polyQ protein and inhibits the function of transcription factors and coactivators as well as other cellular operations (Katsuno et al., 2006; Kemp et al., 2011). A recent study demonstrated deficiencies of retrograde transport in spinal motor neurons in both a knock-in and a myogenic transgenic mouse model of SBMA, implying that disease triggered in muscle can impair retrograde transport of cargo in motor neuron axons (Kemp et al., 2011). In the same study, researchers found that treatment of diseased muscles with vascular endothelial growth factor reversed the deficit in transport, further supporting the hypothesis of impaired retrograde signaling (Kemp et al., 2011). In another mouse model of SBMA, researchers noted accumulation of neurofilament-H (NF-H), a major component of the cytoskeleton, and synaptophysin (SYN), an integral membrane glycoprotein occurring in presynaptic vesicles of neurons, similar to the intramuscular accumulation observed in skeletal muscle of SBMA patients (Katsuno et al., 2006). In this transgenic mouse model carrying human pathogenic AR, researchers utilized Fluoro-Gold™ labeling and sciatic nerve ligation protocols to demonstrate impaired retrograde axonal transport (Katsuno et al.,
Additionally, significant reductions in mRNA levels of dynactin 1 (DCTN1), resulting from pathogenic AR-induced transcriptional dysregulation, was recorded in the SBMA mice (Katsuno et al., 2006). Researchers reported that castration prevented nuclear accumulation of pathogenic AR, thus reversing the pathological events mentioned previously (Katsuno et al., 2006).

Amyotrophic lateral sclerosis (ALS) is yet another neurodegenerative disease with potential underlying impairments in axonal transport. There are two categories of ALS. Familial ALS (fALS) is caused by heritable factors and accounts for roughly 10% of all cases, whereas sporadic ALS (sALS) has no known causes and seemingly arises randomly. Both types produce similar pathological markers, including progressive muscle weakness, atrophy, and spasticity due to degeneration and death of upper and lower motor neurons (Boillée et al., 2006). Denervation of the respiratory muscles and diaphragm is typically the cause of death (Boillée et al., 2006). The selective vulnerability of motor neurons in ALS has been suggested to be a combination of several factors including protein misfolding, oxidative stress, excitotoxicity, impaired axonal transport, mitochondrial dysfunction, inflammation, and neurotrophin deprivation (Jiang et al., 2005; Boillée et al., 2006). Non-neuronal neighboring cells further enhance damage within motor neurons via inflammatory responses that accelerate disease progression (Boillée et al., 2006).

In both forms of ALS, defective axonal transport is a prime candidate for the pathogenesis observed in human patients and animal models (Jiang et al., 2005; Bilsland et al., 2010). Approximately 20-30% of fALS cases are linked to mutations in the copper/zinc superoxide dismutase 1 (SOD1) gene (Jiang et al., 2005). There are over one
hundred known disease-causing *SOD1* mutations distributed throughout all five exons, and with few exceptions, all mutations are dominant (Boillée et al., 2006). Mice and rats expressing mutant forms of human or mouse SOD1 exhibit progressive motor neuron degeneration and serve as useful animal models for ALS, as fALS and sALS are pathologically and clinically similar (Bruijn et al., 2004; Boillée et al., 2006). SOD1 is a cytosolic enzyme that converts superoxides to water or hydrogen peroxide, and is both abundantly and ubiquitously expressed (Boillée et al., 2006). Surprisingly, it is not the loss of SOD1 function that confers toxicity to mutants, as deletion of the *SOD1* gene in mice does not lead to motor neuron disease (Reaume et al., 1996). Rather, the disease-associated mutations are known to destabilize the protein and cause it to become misfolded (Boillée et al., 2006; Sau et al., 2007). Misfolded SOD1 triggers a cascade of events, including protein accumulation, axonal transport alterations, and mitochondrial and/or proteasome dysfunctions (Sau et al., 2007). These events may indirectly lead to overproduction of reactive oxygen species and caspase activation, and may be intercorrelated (Sau et al., 2007).

An early study by Williamson and Cleveland demonstrated that the reduction of axonal transport is a very early feature of the toxicity in motor neurons mediated by ALS-linked *SOD1* mutants (Williamson and Cleveland, 1999). More recently, Bilsland and colleagues emphasized the presymptomatic appearance of deficits in axonal transport using an *in vivo* assay that permitted characterization of axonal transport in single axons of intact sciatic nerves (Bilsland et al., 2010). ALS patients and mice expressing mutant SOD1 have been shown to display neurofilament accumulations, which are suggested to arise from defects in slow anterograde axonal transport (Boillée et al., 2006; Bilsland et
human mutant SOD1\textsuperscript{G93A} have been shown to exhibit variations in fast axonal transport (Boillée et al., 2006; Bilsland et al., 2010). The studies conducted by Bilsland and associates revealed significant slowing of retrograde transport at each disease stage (presymptomatic, early symptomatic, symptomatic, late symptomatic) in SOD1\textsuperscript{G93A} mice compared to wild type (WT) and SOD1\textsuperscript{WT} mice (Bilsland et al., 2010). Additionally, there was significantly reduced TrkB accumulation in ligated sciatic nerve of SOD1\textsuperscript{G93A} mice compared to WT controls (Bilsland et al., 2010).

\textit{Ottem laboratory axonal transport studies}

The Ottem laboratory, building on previous research, has conducted a set of experiments delving into potential axonal transport defects in transgenic mice missing muscle-synthesized BDNF (Dangremond, 2016). In these experiments, based on a protocol by Katsuno et al., 2006, sciatic nerve ligations were conducted on 30d and 120d homozygous knockout (Muscle\textsuperscript{BDNF-/-}), heterozygous knockout (Muscle\textsuperscript{BDNF+/-}), and wild type (Muscle\textsuperscript{BDNF+/-}) animals (Dangremond, 2016). In this experiment, accumulation of three proteins of interest were evaluated at the areas immediately proximal and distal to the ligation site. The labeled proteins were dynactin, SYN, and phosphorylated NF-H (NF-H-P). The structural protein NF-H is delivered from the cell soma to the distal axon in vesicles by the motor protein kinesin. As NF-H is transported anterogradely down the axon, it gets increasingly phosphorylated, which diminishes the protein’s affinity for kinesin and enhances its affinity for dynein (Motil et al., 2007). Thus, NF-H was expected to associate with kinesin motors in anterograde transport complexes, and was
not investigated. Alternatively, NF-H-P was hypothesized to be associated with dynactin in retrograde transport complexes, and was labeled as a protein of interest in this experiment.

Assessment of NF-H-P accumulation on the distal side of the ligation site in Muscle$^{BDNF+/+}$, Muscle$^{BDNF+/-}$, and Muscle$^{BDNF-/-}$ animals revealed no significant differences in 30d mice, while there were significant reductions in NF-H-P accumulation in Muscle$^{BDNF-/-}$ animals compared to others at 120d (Figure 14; Dangremond, 2016). Similarly, aggregation of dynactin on the distal side of the ligation site was significantly diminished in Muscle$^{BDNF-/-}$ mice compared to control at 120d (Figure 15; Dangremond, 2016). There were no significant differences in the accumulation of SYN between groups in 30d and 120d animals (Figure 16; Dangremond, 2016).

![Figure 14. Mean surface area (µm$^2$) of phosphorylated neurofilament-H (NF-H-P) immunofluorescence immediately distal to sciatic nerve ligation site in 30d and 120d mice. (A) At 30d, there were no significant differences in mean NF-H-P surface area between Muscle$^{BDNF+/+}$, Muscle$^{BDNF+/-}$, or Muscle$^{BDNF-/-}$ mice. (B) At 120d, only homozygous knockouts exhibited significant reductions in mean NF-H-P immunofluorescence surface area compared to controls (** = p<0.01). Adapted from Dangremond (2016).]
Figure 15. Mean surface area (µm$^2$) of dynactin immunofluorescence in the portion immediately distal to sciatic nerve ligation site in 120d Muscle$^{BDNF+/+}$, Muscle$^{BDNF+/−}$, and Muscle$^{BDNF−/−}$ mice. There were significant reductions only in homozygous knockouts compared to control animals (** = p<0.01). Adapted from Dangremond (2016).
Figure 16. Mean surface area (µm$^2$) of synaptophysin (SYN) immunofluorescence in portion immediately distal to sciatic nerve ligation site in 30d and 120d Muscle$^{BDNF+/+}$, Muscle$^{BDNF+/-}$, and Muscle$^{BDNF-/-}$ mice. At 30d, no significant differences in SYN surface area were recorded across genotypes, however, there was a trending increase in SYN immunofluorescence. At 120d, there were no significant changes in mean surface area. Adapted from Dangremond (2016).

There were no differences in NF-H-P accumulation on the proximal side of the ligation site between Muscle$^{BDNF+/+}$, Muscle$^{BDNF+/-}$, and Muscle$^{BDNF-/-}$ animals (Figure 17; Dangremond, 2016). Conversely, there were significant increases in dynactin accumulation at the proximal side of the ligation site in Muscle$^{BDNF+/-}$ and Muscle$^{BDNF-/-}$ mice (Figure 18; Dangremond, 2016). Similar to NF-H-P, there were no significant changes in SYN accumulation at the proximal site between animals (Figure 19; Dangremond, 2016).
Figure 17. Mean surface area (µm²) of NF-H-P immunofluorescence immediately proximal to ligation site in 120d Muscle^{BDNF+/+}, Muscle^{BDNF+/−}, and Muscle^{BDNF−/−} mice. There were no significant differences across genotypes. Adapted from Dangremond (2016).
Figure 18. Mean surface area (µm²) of dynactin immunofluorescence immediately proximal to ligation site in 120d Muscle^BDNF+/+, Muscle^BDNF+/−, and Muscle^BDNF−/− mice. Mean surface area of dynactin was significantly increased in heterozygous and homozygous knockout animals compared to controls (* = p<0.05). Adapted from Dangremond (2016).
In addition to sciatic nerve ligation studies, experiments that further investigated the possibility that retrograde transport is diminished in mice missing muscle-synthesized BDNF were conducted (Dangremond, 2016). The Ottem lab assessed levels of NF-H-P – a protein that, under normal cellular conditions, should be associated with dynein and retrogradely transported to the cell body for recycling – in gastroc-associated NMJs of these transgenic mice (Dangremond, 2016). An accumulation of NF-H-P in the axons of gastroc-associated motor neurons would suggest potential deficits in retrograde transport. Indeed, although there were no differences in levels of NF-H-P between Muscle$^{BDNF^{+/+}}$, Muscle$^{BDNF^{+/−}}$, and Muscle$^{BDNF^{−/−}}$ mice at 30d (Figure 20A), there were significant
increases in both the heterozygous and homozygous knockout animals compared to controls at 120d (Figure 20B; Dangremond, 2016).

![Image of bar charts showing mean surface area of NF-H-P immunolabeling](image)

**Figure 20.** Mean surface area ($\mu m^2$) of NF-H-P immunofluorescence in distal gastroc-associated motor neuron axons and presynaptic terminals of 30d and 120d Muscle$^{BDNF+/+}$, Muscle$^{BDNF+/-}$, and Muscle$^{BDNF-/-}$ mice. (A) No significant differences in NF-H-P immunolabeling were observed in 30d animals. (B) At 120d, heterozygous and homozygous knockouts exhibited significant increases in mean NF-H-P surface area compared to controls (** = p<0.01). Adapted from Dangremond (2016).

**Experimental aims**

Given that dynactin accumulation on the distal sciatic ligation site is reduced in Muscle$^{BDNF-/-}$ animals (Figure 15) and NF-H-P accumulation is increased in both 120d Muscle$^{BDNF+/+}$ and Muscle$^{BDNF-/-}$ mice (Figure 20B), it would appear that mice missing muscle-synthesized BDNF have disruptions in retrograde transport (Dangremond, 2016). Because of the prevalence of retrograde transport dysfunction in neurodegenerative diseases such as ALS, and the pathological similarities of these mice to other animal models of neurodegenerative diseases, the goal of present studies was to further
investigate and characterize the retrograde transport deficits recorded in these mice (Katsuno et al., 2006; Moser et al., 2013; Dangremond, 2016). As BDNF-TrkB signaling has been shown to play such a critical role in motor neuron health and axonal transport, the expression of TrkB and its activated form, p-TrkB, was assessed in gastroc-associated NMJs of 120d Muscle$^{BDNF+/+}$, Muscle$^{BDNF+/-}$, and Muscle$^{BDNF-/-}$ mice (Ginty and Segal, 2002; Mitchell et al., 2012). Additionally, a sciatic nerve ligation protocol (adapted from Katsuno et al., 2006 and Dangremond, 2016) was used to evaluate the composition and density of axonal transport complexes in 120d mice missing muscle-synthesized BDNF to further address the retrograde transport deficits reported in these animals (Dangremond, 2016). IHC was utilized to label JIP3, an adaptor protein, α-tubulin, a structural protein, dynactin, TrkB, and p-TrkB in ligated sciatic nerves.
CHAPTER TWO: GENERATION OF EXPERIMENTAL TRANSGENIC ANIMALS

Introduction

For all experiments, the Cre/lox system was used to generate tissue-specific BDNF gene knockout animals. To accomplish this, two separate colonies of transgenic mice, originally obtained from Jackson Laboratories (Bar Harbor, Maine), were maintained. In the first strain, the coding region of the BDNF gene was flanked by two 34bp loxP sequences (floxed BDNF), thus providing the recognition sites for the Cre recombinase protein. The second colony carried the Cre transgene driven by the human skeletal actin promoter on the X-chromosome, and consequently, only expressed Cre recombinase in skeletal muscle cells. Crossing and back-crossing offspring from the two colonies results in transgenic mice wherein BDNF is knocked out only in skeletal muscle. See Figure 21 for a schematic description of the transgenic crossing system. Figure 22 depicts the polymerase chain reaction (PCR) results from various tissue types confirming the loss of BDNF only in skeletal muscle in these transgenic animals. For each experiment, a group of wild-type control (MuscleBDNF+/+), heterozygous knockout (MuscleBDNF+/−), and homozygous knockout (MuscleBDNF−/−) animals were used.
Figure 21. Diagram detailing the tissue-specific targeting of BDNF in skeletal muscle using the Cre/lox system. A mouse carrying the Cre recombinase transgene driven by the human skeletal actin promoter is crossed with a mouse that has BDNF flanked by loxP recognition sites (floxed BDNF). Because of the specificity of the human skeletal promoter, the Cre recombinase enzyme is only expressed in skeletal muscle. Thus, animals positive for both the Cre transgene and floxed BDNF will have the coding region of the BDNF gene (exon 9) excised only in skeletal muscle. See Figure 22 for PCR confirmation of muscle-specific loss of BDNF in skeletal muscle in these transgenic mice.
Figure 22. Results of BDNF gene PCR assay confirming loss of BDNF in skeletal muscle using the Cre/lox system. PCR amplification products were electrophoresed on an agarose gel, stained with ethidium bromide, and visualized with UV light. (A) Control (Muscle$^{BDNF+/+}$) mice had the full-length coding region of BDNF (2050bp) amplified in skeletal muscle, kidney, heart, and brain tissue. (B) The BDNF amplification product is truncated (975bp) only in the skeletal muscle of homozygous knockout mice (Muscle$^{BDNF/-}$).

Methods

Animal husbandry

Animals were maintained according to the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee (IACUC) of Northern Michigan University (Appendix A). Animals were housed in the temperature- and light-controlled animal facility at
Northern Michigan University with a 14:10 hour light-dark schedule (lights on from 07:00 to 21:00) and had access to food (Mazuri® Rodent Chow, Land O’ Lakes Purina Feed LLC, Richmond, IN) and water *ad libitum*.

*Genotyping of experimental animals*

Each animal was earpunched for identification, and the resultant tissue was used for genotyping procedures. Genotyping was accomplished through the use of two separate PCR reactions – one for the detection of the floxed *BDNF* gene and one for the presence of the *Cre* transgene. Visualization of the PCR amplification products was attained using agarose gel electrophoresis. Two primers were employed in the PCR used to verify the presence (or lack) of floxed *BDNF* alleles in each animal. The forward (1846) primer sequence was TGTGATTGTGTTTCTGGTGAC, and the reverse (1847) primer sequence was GCCTTCATGCAACCGAAGTATG. The 5’ forward primer hybridized to a segment upstream of the first *loxP* site, and the 3’ reverse primer hybridized to a segment internal to the *BDNF* gene coding region. The use of these two primers allowed for amplification of both floxed and non-floxed *BDNF*. The resulting amplification products were differentiated based on size: floxed *BDNF* products were 487bp whereas non-floxed were 419bp (shown in Figure 23).

A different set of primers was employed in the PCR evaluating whether the *Cre* transgene was present or absent in each animal. The forward (1084) primer sequence for the *Cre* transgene was GCGGTCTGGCAGTAAAAACTATC. The reverse (1085) primer sequence was GTGAAACAGCATTGCTGTCACTT. In addition, another pair of primers specific for a housekeeping gene (*interleukin 2, IL2*) were used as a positive control.
alongside the pair of primers targeting the Cre transgene. The IL2 primer sequences used were CTAGGCCACAGAATTGAAAGATCT (forward; 7338), and GTAGGTGGAAATTCTAGCATCATCC (reverse; 7339). After amplification, the PCR products were visualized on an agarose gel, with all animals displaying a 300bp band representing the IL2 housekeeping gene, while only those animals heterozygous for the Cre transgene show a 100bp amplification band. An example of typical results for the Cre transgene PCR can be found below in Figure 24.

Figure 23. Photograph of BDNF gene PCR amplification products ran on 3% agarose gel, stained with ethidium bromide, and visualized using UV light. The first lane displays a 100bp ladder (Axygen® 100bp DNA ladder, product #M-DNA-100bp, Corning, Inc.). In the remaining lanes, the upper band (487bp) represents floxed BDNF, while the lower band (419bp) is non-floxed BDNF.
Figure 24. Photograph of Cre transgene PCR amplification products electrophoresed on a 1% agarose gel and stained with ethidium bromide, visualized using UV light. The first lane contains a 100bp ladder (Axygen® 100bp DNA ladder, product #M-DNA-100bp, Corning, Inc.). In the other lanes, the upper band (300bp) corresponds to amplification of a housekeeping gene (IL2) all animals have, while the lower band (100bp) represents the Cre transgene.

Breeding scheme for heterozygous knockout animals

After determining genotypes using the PCR techniques described above in Figures 23 and 24, animals with loxP-flanked (floxed) BDNF (BDNF\textsuperscript{loxP/+, Cre\textsuperscript{-/-}}) were crossed with animals expressing the Cre transgene (BDNF\textsuperscript{loxP/-, Cre\textsuperscript{+/-}; F1 crosses, Figure 25}). The F1 offspring derived from these crosses were either heterozygous for both the floxed BDNF and Cre recombinase transgene (BDNF\textsuperscript{loxP/+, Cre\textsuperscript{+/-}}) or heterozygous for the floxed BDNF and missing Cre (BDNF\textsuperscript{loxP/+, Cre\textsuperscript{-/-}}). Males that were heterozygous for both floxed BDNF and Cre (BDNF\textsuperscript{loxP/+, Cre\textsuperscript{+/-}; referred to throughout as Muscle\textsuperscript{BDNF+/+}} mice) were used as heterozygous knockouts in experiments, while the females of this genotype (BDNF\textsuperscript{loxP/+, Cre\textsuperscript{+/-}}) were used as F2 breeders to generate control and homozygous knockout animals. See Figure 25 for reference.
Breeding of homozygous knockout and control mice

Females heterozygous for floxed BDNF and the Cre transgene (BDNF\textsuperscript{loxP+/−}, Cre\textsuperscript{+/−}) were crossed with males expressing floxed BDNF (BDNF\textsuperscript{loxP+/+}, Cre\textsuperscript{−/−}). The resulting male F2 offspring homozygous for floxed BDNF and heterozygous for the Cre transgene (BDNF\textsuperscript{loxP+/+}, Cre\textsuperscript{+/−}; referred to throughout as Muscle\textsuperscript{BDNF−/−}) were used as homozygous knockout animals in experiments, while those homozygous for floxed BDNF and lacking the Cre transgene (BDNF\textsuperscript{loxP+/+}, Cre\textsuperscript{−/−}; referred to throughout as Muscle\textsuperscript{BDNF+/+}) were used as control animals. See Figure 25 below for reference.
Figure 25. Diagram of the breeding scheme used to generate experimental animals. In the F1 crosses, floxed BDNF mice (BDNF$^{loxP+/+}$, Cre$^{-/-}$) were mated with mice carrying the Cre transgene (BDNF$^{loxP-/-}$, Cre$^{+/-}$) to generate heterozygous knockout (HE KO) animals (MuscleBDNF$^{+/-}$, circled in purple). Heterozygous KO males were used in experiments, while the females were used in F2 crosses with floxed BDNF males to generate homozygous knockout (HO KO) animals (MuscleBDNF$^{-/-}$, circled in red) and wild-type control (CO) animals (MuscleBDNF$^{+/+}$, circled in blue).
CHAPTER THREE: ASSESSMENT OF TRKB AND P-TRKB EXPRESSION IN GASTROC-ASSOCIATED NMJS OF MICE MISSING MUSCLE-SYNTHESIZED BDNF

Introduction

Neurotrophins are important for the development, maintenance, and survival of neurons, and their actions are mediated by Trk receptors (Huang and Reichardt, 2001). Of particular interest is BDNF activation of TrkB, which is important in the initiation of retrograde transport (Mitchell et al., 2012). Specifically, this ligand-receptor interaction recruits cytoplasmic dynein to signaling endosomes for retrograde axonal transport (Ginty and Segal, 2002; Mitchell et al., 2012). Mice missing muscle-synthesized BDNF display a number of myopathological features such as hyper- and hypotrophic fibers, fiber splitting, centralized nuclei, age-independent loss of muscle fibers, and gastroc-associated NMJ fragmentation (Pomeroy, 2013; Dangremond, 2016). These transgenic mice also exhibited reduced dendritic Fluoro-Gold™ labeling in gastroc-associated lumbar motor neurons (Pomeroy, 2013).

Studies attempting to determine whether reduced dendritic labeling was due to dendritic atrophy or reduced retrograde transport focused on NF-H-P, a protein ubiquitously transported retrogradely to the cell body for recycling (Dangremond, 2016). These experiments found significant accumulation of NF-H-P in the axons and presynaptic terminals of gastroc-associated motor neurons in transgenic mice missing muscle-synthesized BDNF (Figure 20; Dangremond, 2016). These results suggested that disruption of retrograde transport, rather than dendritic atrophy, accounted for reduced Fluoro-Gold™ labeling. Further, sciatic nerve ligation studies revealed significantly
decreased accumulation of both NF-H-P and dynactin on the distal side of the ligation site in these transgenic mice (Figures 14 and 15, respectively; Dangremond, 2016). Together, these experiments suggest that mice-missing muscle-synthesized BDNF have deficits in retrograde transport.

As BDNF-TrkB signaling is important for initiating the formation of retrograde transport complexes, the first aim of this study was to assess the expression and phosphorylation state of TrkB in gastroc-associated NMJs. To do this, antibodies against TrkB and its activated form, phosphorylated-TrkB (p-TrkB), were employed alongside antibodies targeting vesicular acetylcholine transporter (VACHT) to label the presynapse, and α-BTX to label the acetylcholine receptors (AChR) in the postsynapse.

Methods

Animals

Animals in this experiment were maintained according to the NIH Guidelines for the Care and Use of Laboratory Animals and approved by the IACUC of Northern Michigan University (Appendix A). A total of 22 male mice at 120d of age were used in these studies because all previous Ottem laboratory studies utilized males. Mice were bred from existing colonies originally purchased from Jackson Laboratories (Bar Harbor, Maine). For this experiment, seven wild type (WT) control (MuscleBDNF+/+) mice, eight heterozygous knockout (MuscleBDNF+/-) mice, and seven homozygous knockout (MuscleBDNF-/-) mice were used. Animals were housed in the temperature- and light-controlled animal facility at Northern Michigan University with a 14:10 hour light-dark
schedule (lights on from 07:00 to 21:00), and had access to food (Mazuri® Rodent Chow, Land O’ Lakes Purina Feed LLC, Richmond, IN) and water *ad libitum*.

*Tissue harvest and processing*

At age 120d, mice were anesthetized via inhalation of IsoThesia™ (isoflurane, Henry Schein® Animal Health, Dublin, OH), and the gastroc muscle was removed from the left leg. The muscle was flash frozen, mounted on Tissue-Tek® OCT compound (Sakura® Finetek USA, Inc., Torrance, CA), and kept on dry ice until storage at -80 °C. Immediately after the gastroc was harvested, mice were euthanized using an overdose of sodium pentobarbital (250 mg/kg; Sigma-Aldrich®, St. Louis, MO) via intraperitoneal (IP) injection. Muscles were sectioned longitudinally at 40 µm using a Leica CM1850 Cryostat and mounted onto subbed slides, then alternately processed using immunohistochemical assays to label for TrkB or p-TrkB. This tissue harvest protocol was used for roughly half of the animals (n=3) in each group.

*Alternate method of tissue harvest and processing*

At 120d of age, mice were anesthetized via inhalation of IsoThesia™ (isoflurane, Henry Schein® Animal Health, Dublin, OH) and euthanized via IP injection of sodium pentobarbital (250 mg/kg; Sigma-Aldrich®, St. Louis, MO). Tissues were fixed via intracardial perfusion with 25 mM phosphate-buffered saline (PBS; 3 mM monobasic sodium phosphate, 22.1 mM dibasic sodium phosphate, 152.3 mM sodium chloride) followed by 4% paraformaldehyde (in 25 mM PBS). Following fixation, the left gastroc muscle was removed, flash frozen, mounted on Tissue-Tek® OCT compound (Sakura®
Finetek USA, Inc., Torrance, CA), and stored at -80 °C. Muscles were sectioned longitudinally at 40 µm using a Leica CM1850 Cryostat and mounted onto subbed slides, then alternately processed using immunohistochemical assays to assess TrkB or p-TrkB expression. This tissue harvest protocol was used for half of the animals (n=4) in each group, and yielded superior sectioning results.

**TrkB immunohistochemistry (IHC)**

To visualize TrkB expression at the NMJ, muscle sections were chemically cross-linked to subbed slides by incubation in paraformaldehyde (4% in 1:5 0.2 M monobasic: 0.2 M dibasic sodium phosphate buffer, pH 7.4) for 15 min and then rinsed with 1X PBS (140 mM sodium chloride, 2.7 mM potassium chloride, 1.5 mM monobasic potassium phosphate, 8 mM dibasic sodium phosphate heptahydrate). After incubating 1 hour with blocking buffer (10% normal donkey serum, 0.2% Triton® X-100, 0.01% sodium azide in 1X PBS), the tissue was incubated overnight with anti-TrkB (1:500, rabbit polyclonal antibody, ab18987; Abcam, Cambridge, MA) and anti-VACht (1:500, goat polyclonal antibody, sc-7717; Santa Cruz® Biotechnology, Inc., Dallas, TX) in blocking buffer. The tissue was washed with 1X PBS and incubated for 1 hour with the secondary antibody solution (1X PBS, 0.01% sodium azide, 0.2% Triton® X-100, and secondary antibodies indicated in Table 1). The tissue was rinsed with 1X PBS, allowed to dry, then coverslipped using Vectasheild® Mounting Medium with DAPI (Vector Laboratories, Inc., Burlingame, CA) to prevent quenching, and slides were stored in the dark at 4 °C.
Three different secondary antibody labeling schemes were used for gastroc TrkB IHC. Product numbers are included with each labeling fluorophore. All α-BTX secondaries were used at 1:200 and obtained from Molecular Probes® Life Technologies™ (Eugene, OR). All anti-rabbit and anti-goat secondary antibodies were used at 1:150 and purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

### Table 1. Different secondary antibody labeling schemes used for TrkB gastroc IHC.

<table>
<thead>
<tr>
<th>Fluorescent dye</th>
<th>Secondary preparation #1</th>
<th>Secondary preparation #2</th>
<th>Secondary preparation #3</th>
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</thead>
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<td>Donkey anti-rabbit (711-545-152)</td>
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<tr>
<td>Alexa Fluor® 594</td>
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<td>Donkey anti-goat (705-605-003)</td>
<td>Donkey anti-goat (705-605-003)</td>
<td>α-BTX (B35450)</td>
</tr>
</tbody>
</table>

*p-TrkB IHC*

To visualize p-TrkB expression at the NMJ, muscle sections were chemically cross-linked to subbed slides by incubation in paraformaldehyde (4% in 0.2 M phosphate buffer, pH 7.4) for 15 min and then rinsed with 1X PBS. After incubating 1 hour with blocking buffer (10% normal donkey serum, 0.2% Triton® X-100, 0.01% sodium azide in 1X PBS), the tissue was incubated overnight with anti-p-TrkB (1:500, Tyr816, rabbit polyclonal antibody, ABN1381; MilliporeSigma, Temecula, CA) and anti-VACHT (1:500, goat polyclonal antibody, sc-7717; Santa Cruz® Biotechnology, Inc., Dallas, TX) in blocking buffer. The tissue was washed with 1X PBS and incubated for 1 hour with the secondary antibody solution (1X PBS, 0.01% sodium azide, 0.2% Triton® X-100, and secondary antibodies shown below in Table 2). The tissue was rinsed with 1X PBS, allowed to dry, then coverslipped using Vectasheild® Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA) to prevent quenching, and slides were stored in the dark at 4 °C.
Three different secondary antibody labeling schemes were used for gastroc p-TrkB IHC. Product numbers are included with each labeling fluorophore. All α-BTX secondaries were used at 1:200 and obtained from Molecular Probes® Life Technologies™ (Eugene, OR). All anti-rabbit and anti-goat secondary antibodies were used at 1:150 and purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

**Imaging, data collection, and analysis**

Photomicrographs were collected at 60X using an Olympus Fluoview FV1000 laser scanning confocal microscope. Scanning speed, aspect ratio, and step size were set at 20 µs/pixel, 800x800, and 0.44 µm, respectively. Image X, Y, and Z coordinate limits were adjusted to include entire neuromuscular junctions in the final Z-stack. A minimum of 45 junctions were imaged for each animal in the three experimental groups.

Photomicrographs were uploaded into Bitplane’s Imaris 3D rendering computer software (Concord, MA) and, using the surface tool, 3D surfaces were created for the α-BTX, VAChT, and TrkB (or p-TrkB) immunofluorescent channels (post-synapse, pre-synapse, and receptor localization, respectively). Colocalization between TrkB and VAChT surfaces, and TrkB and α-BTX surfaces, were rendered and exported as number of voxels per NMJ. Colocalization between p-TrkB and VAChT surfaces, and p-TrkB and α-BTX surfaces, were generated and quantified as number of voxels per NMJ. Examples of the rendered immunofluorescence and colocalization surfaces for TrkB and p-TrkB can be
found in Figures 26 and 27, respectively. One-way analysis of variance (ANOVA) and post hoc Bonferroni procedure t-tests were used to compare data for the groups.

Figure 26. Assessment of TrkB expression in gastroc-associated NMJs of 120d Muscle$^{BDNF+/+}$ animal. (A) Representative confocal photomicrograph (60X) showing TrkB (green), VACHT (red), and AChR labeled with α-BTX (cyan) expression in gastroc tissue. (B) Imaris (Bitplane) rendered surfaces for TrkB (green), VACHT (red), and AChR (cyan) expression. (C) Colocalization of TrkB and VACHT in the presynaptic terminals of NMJs. (D) Colocalization of TrkB with AChR in the postsynaptic terminals of NMJs.
Figure 27. Evaluation of p-TrkB expression in gastroc-associated NMJs of 120d Muscle\textsuperscript{BDNF\textsuperscript{-/-}} animal. (A) Representative confocal photomicrograph (60X) showing p-TrkB (green), VAChT (red), and AChR labeled with α-BTX (cyan) expression in gastroc tissue. (B) Imaris (Bitplane) rendered surfaces for p-TrkB (green), VAChT (red), and AChR (cyan) expression. (C) Colocalization of p-TrkB and VAChT in the presynaptic terminals of NMJs. (D) Colocalization of TrkB with AChR in the postsynaptic terminals of NMJs.
Results

TrkB results

In order to assess potential mechanisms underlying apparent retrograde transport deficits in Muscle\textsuperscript{BDNF+/-} and Muscle\textsuperscript{BDNF-/-} mice, first, the levels of TrkB expression in gastrocnemius-associated NMJs of 120d animals were determined. To do this, colocalization of the receptor with VACHT in the presynapse, and α-BTX in the postsynapse, was measured and compared between groups.

One-way ANOVA indicated that the means for TrkB-VACHT colocalization were significantly different \([p = 0.0377, \text{degrees of freedom (df)} = 2, 21, F = 3.916]\). However, use of Bonferroni’s procedure showed that there were no significant differences in the levels of presynaptic TrkB between groups (Figure 28): Muscle\textsuperscript{BDNF+/-} versus Muscle\textsuperscript{BDNF4+/-} \((t = 0.09292, p > 0.05)\), Muscle\textsuperscript{BDNF+/-} versus Muscle\textsuperscript{BDNF-/-} \((t = 2.347, p > 0.05)\), or Muscle\textsuperscript{BDNF+/-} versus Muscle\textsuperscript{BDNF-/-} \((t = 2.517, p > 0.05)\). Although presynaptic TrkB was no different between Muscle\textsuperscript{BDNF+/-} and Muscle\textsuperscript{BDNF4+/-} mice, receptor expression in the NMJ of Muscle\textsuperscript{BDNF-/-} animals appears to be half that of the other two genotypes (Figure 28).

One-way ANOVA of postsynaptic TrkB revealed significantly different means \((p = 0.0016, \text{df} = 2, 20, F = 9.346)\). Bonferroni’s procedure indicated significant differences in the knockout groups compared to controls (Figure 29): Muscle\textsuperscript{BDNF+/-} versus Muscle\textsuperscript{BDNF4+/-} \((t = 3.414, p < 0.01)\) and Muscle\textsuperscript{BDNF+/-} versus Muscle\textsuperscript{BDNF-/-} \((t = 4.004, p < 0.01)\). Whereas there were no significant differences between heterozygous and homozygous knockouts (Figure 29): Muscle\textsuperscript{BDNF+/-} versus Muscle\textsuperscript{BDNF-/-} \((t = 0.5900, p > 0.05)\). Thus, both Muscle\textsuperscript{BDNF4+/-} and Muscle\textsuperscript{BDNF-/-} animals exhibited significant
reductions in postsynaptic TrkB immunofluorescence compared to control Muscle$^{BDNF^{+/+}}$ mice (Figure 29).

Figure 28. TrkB immunofluorescence associated with VACHT-labeled presynaptic NMJs in 120d mice. There were no significant differences in presynaptic TrkB expression between groups. The variation in TrkB levels were relatively high in this assay; however, TrkB appears to be much lower in Muscle$^{BDNF^{-/-}}$ animals (n=7) compared to Muscle$^{BDNF^{+/+}}$ (n=7) and Muscle$^{BDNF^{+/-}}$ mice (n=8).
Figure 29. TrkB immunofluorescence associated with α-BTX-labeled postsynaptic NMJs in 120d mice. There were significant reductions in postsynaptic TrkB expression in Muscle^{BDNF+/-} and Muscle^{BDNF-/-} mice compared to control animals (n=7; ** = p<0.01). There was no significant difference in TrkB immunofluorescence between Muscle^{BDNF+/-} and Muscle^{BDNF-/-} knockout animals.

p-TrkB results

To further evaluate the mechanisms underlying axonal transport disruptions in Muscle^{BDNF+/-} and Muscle^{BDNF-/-} animals, the levels of the activated TrkB receptor were assessed in gastroc-associated NMJs of 120d mice. Colocalization of p-TrkB with VACHT in the presynaptic terminal, and α-BTX in the postsynaptic terminal, was measured and compared between genotypes.

One-way ANOVA of presynaptic p-TrkB revealed significantly different means (p < 0.0001, df = 2, 21, F = 15.69). Bonferroni’s procedure indicated significant differences in the knockout groups compared to controls (Figure 30): Muscle^{BDNF+/-} versus Muscle^{BDNF+/-} (t = 3.657, p < 0.01) and Muscle^{BDNF+/-} versus Muscle^{BDNF-/-} (t = 5.523, p < 0.001). However, there were no significant differences between heterozygous and homozygous knockouts (Figure 30): Muscle^{BDNF+/-} versus Muscle^{BDNF-/-} (t = 2.047, p
Thus, presynaptic p-TrkB expression was found to be significantly lower in Muscle^{BDNF+/+} and Muscle^{BDNF-/-} animals when compared to control mice (Figure 30).

One-way ANOVA of postsynaptic p-TrkB showed significantly different means ($p = 0.0017$, $df = 2, 21, F = 9.105$). Again, Bonferroni’s t-test indicated significant differences in the knockout groups compared to controls (Figure 31): Muscle^{BDNF+/+} versus Muscle^{BDNF-/-} ($t = 3.388$, $p < 0.01$) and Muscle^{BDNF+/+} versus Muscle^{BDNF-/-} ($t = 3.971$, $p < 0.01$). And once more, there were no significant differences between knockout mice (Figure 31): Muscle^{BDNF+/+} versus Muscle^{BDNF-/-} ($t = 0.7133$, $p > 0.05$). Thus, postsynaptic p-TrkB was significantly reduced in Muscle^{BDNF+/+} and Muscle^{BDNF-/-} mice when compared to controls (Figure 31).

Figure 30. p-TrkB immunofluorescence associated with VACHT-labeled presynaptic NMJs in 120d mice. Muscle^{BDNF+/+} animals (n=8) had significantly reduced presynaptic p-TrkB immunofluorescence compared to controls (n=7; ** = $p<0.01$). Muscle^{BDNF-/-} mice (n=7) also had significantly reduced presynaptic p-TrkB expression compared to WT mice (** = $p<0.001$). There was no significant difference in p-TrkB immunofluorescence between Muscle^{BDNF+/+} and Muscle^{BDNF-/-} knockout animals.
Figure 31. p-TrkB immunofluorescence associated with α-BTX-labeled postsynaptic NMJs in 120d mice. Muscle\textsuperscript{BDNF+/−} animals (n=8) had significantly reduced postsynaptic p-TrkB immunofluorescence compared to controls (n=7; ** = p<0.01). Muscle\textsuperscript{BDNF−/−} mice (n=7) also had significantly reduced postsynaptic p-TrkB expression compared to WT mice (** = p<0.01). There was no significant change in postsynaptic p-TrkB immunofluorescence between Muscle\textsuperscript{BDNF+/+} and Muscle\textsuperscript{BDNF−/−} animals.

Discussion

The expression of TrkB, and its activated form, p-TrkB, were evaluated in the pre- and postsynaptic terminals of gastroc-associated NMJs of 120d mice. There were no significant changes in the levels of presynaptic TrkB across genotypes, however, expression in Muscle\textsuperscript{BDNF−/−} animals was greatly reduced compared to other groups (Figure 28). The equivalent levels of presynaptic TrkB immunofluorescence in Muscle\textsuperscript{BDNF+/+} and Muscle\textsuperscript{BDNF−/−} mice may be due to some type of compensatory mechanism which accounts for the reduced, but not absent, levels of muscle-derived BDNF in Muscle\textsuperscript{BDNF−/−} animals. These heterozygous animals exhibit significantly increased BDNF immunofluorescence at the cell soma of gastroc-associated motor neurons (Figure 8B), and BDNF has been shown to be transported anterogradely from
neuron cell bodies to their terminals (Altar et al., 1997; Pomeroy, 2013). Additionally, BDNF has been shown to trigger membrane insertion of TrkB, and BDNF-TrkB signaling promotes anterograde axonal transport of TrkB, further supporting the consistent levels of presynaptic TrkB between WT and Muscle$^{\text{BDNF}+/\text{-}}$ mice (Cheng et al., 2011).

In the postsynapse, TrkB expression was significantly diminished in both Muscle$^{\text{BDNF}+/\text{-}}$ and Muscle$^{\text{BDNF}+/-}$ mice compared to controls (Figure 29). These results were anticipated, as disruption of TrkB signaling and reduced TrkB expression has been shown to induce disassembly and fragmentation of postsynaptic AChR clusters in NMJs of mice (Gonzalez et al., 1999; Kulakowski et al., 2011). The reduced levels of postsynaptic TrkB expression may also explain the previously reported NMJ fragmentation in Muscle$^{\text{BDNF}+/\text{-}}$ and Muscle$^{\text{BDNF}+/-}$ mice (Figures 6 and 7; Taisto et al., 2013).

Evaluation of presynaptic p-TrkB expression revealed significantly reduced levels of immunofluorescence in Muscle$^{\text{BDNF}+/\text{-}}$ and Muscle$^{\text{BDNF}+/-}$ mice compared to controls (Figure 30). The reduction in presynaptic p-TrkB expression in Muscle$^{\text{BDNF}+/\text{-}}$ animals was surprising because, as mentioned previously, these mice appear to exhibit a compensatory mechanism whereby BDNF and TrkB are transported anterogradely from the cell soma (Pomeroy, 2013). It is possible that BDNF from the cell body is not sufficient, or inefficiently transported anterogradely, to adequately activate TrkB to the same degree that muscle-derived BDNF is able to. The reduced levels of presynaptic p-TrkB in Muscle$^{\text{BDNF}+/-}$ mice was expected, as these animals do not appear to exhibit the same compensatory mechanism as heterozygous knockouts (Figure 8B; Pomeroy, 2013).
Postsynaptic p-TrkB expression was found to be significantly reduced in both Muscle$^{BDNF^{+/+}}$ and Muscle$^{BDNF^{--}}$ animals compared to control mice (Figure 31). These results were expected, as it is likely that the combination of reduced or absent muscle-derived BDNF, reduced postsynaptic TrkB expression, and fragmentation of the postsynapse leads to inhibition of TrkB activation.

These results indicate that presynaptic expression of TrkB is not significantly impacted by reduced or absent muscle-synthesized BDNF. However, postsynaptic TrkB expression is significantly decreased because of reduced or absent muscle-derived BDNF. Additionally, both pre- and postsynaptic activation of TrkB is significantly impaired due to reduced or absent muscle-synthesized BDNF, as evidenced by significantly reduced pre- and postsynaptic p-TrkB expression in heterozygous and homozygous knockout animals. Together, these data suggest severe impairments in both pre- and postsynaptic BDNF-TrkB signaling at the NMJ due to reduced or absent muscle-synthesized BDNF.

Because BDNF-TrkB signaling has been shown to play critical roles in axonal transport and motor neuron health, it is important that future studies further investigate the levels of TrkB expression as well as its activation in these transgenic mice. For example, it would be of particular interest to evaluate the overall levels of both TrkB and p-TrkB in gastroc tissue of Muscle$^{BDNF^{+/+}}$, Muscle$^{BDNF^{++}}$, and Muscle$^{BDNF^{--}}$ animals at various ages using enzyme-linked immunosorbent assays or other protein quantification techniques. Additionally, it would be highly beneficial to assess the abundance of TrkB mRNA in gastroc tissue of Muscle$^{BDNF^{+/+}}$, Muscle$^{BDNF^{++}}$, and Muscle$^{BDNF^{--}}$ mice at
varying stages of development using Northern blotting, *in situ* hybridization, or reverse transcription PCR techniques.
CHAPTER FOUR: EVALUATION AND CHARACTERIZATION OF RETROGRADE TRANSPORT IN MICE MISSING MUSCLE-SYNTHESIZED BDNF USING SCIATIC NERVE LIGATIONS

Introduction

Motor neurons have among the furthest reaching axonal projections of any type of neuron, and as such, require an exhaustive system of endogenous and exogenous support to ensure proper function and survival (Morfini et al., 2009; Maday et al., 2014; McKenney et al., 2014; Ghiretti et al., 2016). Perhaps one of the most important facets of these processes is axonal transport, as motor neurons must maintain continual communication and trafficking of proteins, mitochondria, synaptic vesicles, and other cellular components between the cell soma and the dendrites along far reaching axons (Morfini et al., 2009). Interruptions in axonal transport have been cited as causative factors in numerous neuromuscular diseases such as SBMA, HD, and ALS (Morfini et al., 2009). In particular, retrograde transport – a process by which cellular materials are packaged into vesicles in the distal synaptic terminals of neurons and transported toward the cell body – has been characterized as a significant player in these diseases (Goldstein and Yang, 2000; Morfini et al., 2009).

Several studies have shown the importance of neurotrophin signaling through Trk receptors in the initiation of retrograde axonal transport. One such study measured the direct binding of TrkB with the light and intermediate chains of dynein. Investigators observed significant accumulations of TrkB with light and intermediate chains of dynein following sciatic nerve ligation, which suggests an explicit association between TrkB and dynein transport complexes (Yano et al., 2001). Additionally, TrkB activation of the
Erk1/2 kinase pathway recruits cytoplasmic dynein to signaling endosomes for retrograde axonal transport, while inhibition of Erk1/2 negatively affects the motility of TrkB endosomes and their colocalization with dynein in axons (Mitchell et al., 2012). Other studies have reported the importance of the adaptor proteins dynactin (DCTN1) and JIP3 in mediating neurotrophin-Trk receptor signaling and axonal transport (Huang et al., 2011; McKenney et al., 2014; Sun et al., 2017).

Using Fluoro-Gold™ labeling protocols, the Ottem laboratory has demonstrated that 30d and 120d transgenic mice with reduced (MuscleBDNF+/−) or absent (MuscleBDNF−/−) muscle-synthesized BDNF exhibit reductions in the dendritic length of gastroc-associated motor neurons (Pomeroy, 2013). However, as mentioned previously, this may have been due to deficits in retrograde axonal transport rather than abnormalities in motor neuron morphology. Subsequent experiments investigating these potential declines revealed significantly increased accumulation of NF-H-P – a structural protein normally transported retrogradely to the cell body for recycling – in the axons of gastroc-associated motor neurons in MuscleBDNF+/− and MuscleBDNF−/− mice (Figure 20B; Dangremond, 2016). Additionally, following sciatic nerve ligation, 120d MuscleBDNF−/− mice display significantly diminished accumulation of both dynactin and NF-H-P on the distal side of the ligation site (Figures 14B and 15, respectively), suggesting marked retrograde transport dysfunction in these mice (Dangremond, 2016).

Because of the apparent deficits in retrograde transport observed in these transgenic mice, the present study aims to further characterize the nature of transport dysfunction using the same sciatic nerve ligation protocol (adapted from (Katsuno et al., 2006; Dangremond, 2016). If BDNF activation of TrkB at the presynaptic terminal is
required for the initiation of retrograde transport of signaling endosomes, then significant reductions in transport complex accumulation on the distal side of ligation were expected in Muscle^{BDNF+/+} and Muscle^{BDNF-/-} mice when compared to control animals. In order to assess this, following ligation, tissue was immunohistochemically labeled and the expression of TrkB, p-TrkB, JIP3, DCTN1, and α-tubulin was evaluated to determine the composition and accumulation of transport complexes on either side of the ligation site.

The structural protein α-tubulin was included as an internal control, because levels of this protein should not differ proximally or distally between groups or IHC experiments.

**Methods**

**Animals**

Animals in this experiment were maintained according to the *NIH Guidelines for the Care and Use of Laboratory Animals* and approved by the IACUC of Northern Michigan University (Appendix A). A total of 21 male mice at 120d of age were used in these studies. Mice were bred from existing colonies originally purchased from Jackson Laboratories (Bar Harbor, Maine). WT Muscle^{BDNF+/+} control mice (n=7), Muscle^{BDNF+/+} heterozygous knockout mice (n=7), and Muscle^{BDNF-/-} homozygous knockout mice (n=7) were used in these experiments. Animals were housed in the temperature- and light-controlled animal facility at Northern Michigan University with a 14:10 hour light-dark schedule (lights on from 07:00 to 21:00), and had access to food (Mazuri® Rodent Chow, Land O’ Lakes Purina Feed LLC, Richmond, IN) and water *ad libitum.*
Sciatic nerve ligations and tissue harvest and processing

This sciatic nerve ligation protocol was adapted from (Dangremond, 2016) and (Katsuno et al., 2006). One hour prior to surgery, mice were administered the analgesics Metacam® (meloxicam, oral, 5 mg/kg, Boehringer Ingelheim Vetmedica, Inc., Ridgefield, CT) and buprenorphine (subcutaneous, 2.5 mg/kg, Hospira, Inc., Lake Forest, IL). At the time of surgery, mice were anesthetized via inhalation of IsoThesia™ (isoflurane, Henry Schein® Animal Health, Dublin, OH), and the right sciatic nerve was exposed and tied at mid-thigh level with surgical thread (REDILON™, 6-0, 18”; MYCO Medical, Cary, NC). For a control, the left sciatic nerve was exposed at mid-thigh, but not ligated. The left and right incisions were closed using Reflex 7 mm stainless steel wound clips (CellPoint Scientific, Inc., Gaithersburg, MD) and the mice were removed from the isoflurane anesthesia. Animals were monitored for signs of distress every hour. Four hours following surgery, mice were administered a supplemental dose of buprenorphine (subcutaneous, 2.5 mg/kg, Hospira, Inc., Lake Forest, IL). After eight hours, mice were euthanized via IP injection of sodium pentobarbital (250 mg/kg; Sigma-Aldrich®, St. Louis, MO), then perfused with 25 mM PBS followed by 4% paraformaldehyde (in 25 mM PBS). The right sciatic nerve was re-exposed and removed, with at least 5 mm of nerve on both the proximal and distal sides of the ligation site. The left, non-ligated sciatic nerve was harvested in the same manner. The nerves were post-fixed in 4% paraformaldehyde (in 25 mM PBS) for 1 hour, then transferred to ice-cold 20% sucrose (in 25 mM PBS). The extracted sciatic nerves were sectioned at 14 µm using a Leica CM1850 cryostat, mounted onto subbed slides, and alternately processed in two separate
immunohistochemical assays to assess either TrkB or p-TrkB expression within transport complexes.

TrkB IHC

Sciatic nerve tissue sections were chemically crosslinked to subbed slides by incubating for 20 min in 4% paraformaldehyde (in 0.2 M phosphate buffer, pH 7.4). After washing with 1X PBS, nerve sections were placed in blocking buffer (10% normal donkey serum, 0.2% Triton® X-100 and 0.01% sodium azide in 1X PBS) for 1 hour. Tissue was incubated for 48 hours (24 hr RT, 24 hr 4 °C) with the following primary antibodies in blocking buffer: anti-TrkB (1:500, rabbit polyclonal antibody, ab18987; Abcam, Cambridge, MA), anti-JIP3 (1:500, mouse monoclonal antibody, sc-46663; Santa Cruz® Biotechnology, Inc., Dallas, TX), anti-DCTN1 (1:200, goat polyclonal antibody, sc-9801; Santa Cruz® Biotechnology, Inc., Dallas, TX), and anti-α-tubulin (1:200, chicken polyclonal antibody, ab89984; Abcam, Cambridge, MA). Sections were rinsed with 1X PBS and incubated with the secondary antibody solution (0.2% Triton® X-100, 0.01% sodium azide, 1X PBS, and secondary antibodies indicated in Table 3) for 1 hour. Similar to the gastroc IHC, different secondary antibody labeling schemes were used for different slides, and are shown below in Table 3. Nerve sections were washed with 1X PBS, allowed to dry, then coverslipped with ProLong® Gold antifade mounting reagent (Molecular Probes® Life Technologies™, Eugene, OR).
### Table 3. Secondary labeling used for sciatic nerve TrkB IHC.

<table>
<thead>
<tr>
<th>Fluorescent dye</th>
<th>Secondary preparation #1</th>
<th>Secondary preparation #2</th>
<th>Secondary preparation #3</th>
</tr>
</thead>
<tbody>
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<td>Donkey anti-chicken (703-475-155)</td>
<td>Donkey anti-chicken (703-475-155)</td>
</tr>
<tr>
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<td>Donkey anti-mouse (715-545-150)</td>
<td>Donkey anti-rabbit (711-545-152)</td>
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<tr>
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<td>Donkey anti-rabbit (711-585-152)</td>
<td>Donkey anti-mouse (715-585-150)</td>
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<tr>
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<td>Donkey anti-rabbit (711-605-152)</td>
<td>Donkey anti-goat (705-605-003)</td>
<td>Donkey anti-goat (705-605-003)</td>
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Secondary antibody labeling schemes used for TrkB sciatic nerve IHC. Product numbers are included with each secondary. All secondaries were used at 1:200 and obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

**p-TrkB IHC**

Sciatic nerve tissue sections were chemically crosslinked to subbed slides by incubating for 20 min in 4% paraformaldehyde (in 0.2 M phosphate buffer, pH 7.4). After washing with 1X PBS, nerve sections were placed in blocking buffer (10% normal donkey serum, 0.2% Triton® X-100 and 0.01% sodium azide in 1X PBS) for 1 hour. Tissue was incubated for 48 hours (24 hr RT, 24 hr 4 °C) with the following primary antibodies in blocking buffer: anti-p-TrkB (1:500, Tyr816, rabbit polyclonal antibody, ABN1381; MilliporeSigma, Temecula, CA), anti-JIP3 (1:500, mouse monoclonal antibody, sc-46663; Santa Cruz® Biotechnology, Inc., Dallas, TX), anti-DCTN1 (1:200, goat polyclonal antibody, sc-9801; Santa Cruz® Biotechnology, Inc., Dallas, TX), and anti-α-tubulin (1:200, chicken polyclonal antibody, ab89984; Abcam, Cambridge, MA). Sections were rinsed with 1X PBS and incubated with the secondary antibody solution (0.2% Triton® X-100, 0.01% sodium azide, 1X PBS, and secondary antibodies indicated in Table 4) for 1 hour. As with the TrkB sciatic nerve IHC, different secondary antibody labeling schemes were used for different slides, and can be found below in Table 4.
Nerve sections were washed with 1X PBS, allowed to dry, then coverslipped with ProLong® Gold antifade mounting reagent (Molecular Probes® Life Technologies™, Eugene, OR).

Table 4. Secondary antibody labeling used for sciatic nerve p-TrkB IHC.

<table>
<thead>
<tr>
<th>Fluorescent dye</th>
<th>Secondary preparation #1</th>
<th>Secondary preparation #2</th>
<th>Secondary preparation #3</th>
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<tbody>
<tr>
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<td>Donkey anti-chicken (703-475-155)</td>
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<tr>
<td><em>Alexa Fluor® 488</em></td>
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<td><em>Alexa Fluor® 647</em></td>
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<td>Donkey anti-goat (705-605-003)</td>
<td>Donkey anti-goat (705-605-003)</td>
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Secondary antibody labeling schemes used for p-TrkB sciatic nerve IHC. Product numbers are included with each secondary. All secondaries were used at 1:200 and purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

**Imaging, data collection, and analysis**

To assess protein accumulation following ligation, 20 photomicrographs per animal for the TrkB IHC experiment and 20 photomicrographs per animal for the p-TrkB IHC experiment were acquired using an Olympus Fluoview FV1000 confocal laser scanning microscope. For each experiment, these included 10 images of the segment immediately proximal to the ligation site and 10 images of the distal portion.

Photomicrographs were collected at 10X and scanning speed, aspect ratio, digital zoom, and step size were set at 20 μs/pixel, 1024x1024, 1.6, and 4.77 μm, respectively.

Confocal images were uploaded into Bitplane’s Imaris® 3D rendering software for data collection. Separate surfaces were created for each protein (α-tubulin, TrkB or p-TrkB, JIP3, and DCTN1) within 500 μm of the ligation site. Figure 32 shows an example of the
rendered surfaces in the proximal portion of a ligated sciatic nerve. Total surface area (µm²) for each protein was obtained and one-way ANOVA and post-hoc Bonferroni procedure t-test statistical analyses were performed to determine the accumulation of α-tubulin, TrkB or p-TrkB, JIP3, and DCTN1 on the proximal and distal sides of the ligation site in experimental and control animals.

Figure 32. Assessment of protein accumulation on proximal side of ligated sciatic nerve in 120d MuscleBDNF+/− mouse. (A) Representative confocal photomicrograph (10X magnification, 1.6 digital zoom) of the proximal segment of ligated sciatic nerve with an area isolated and measured 500 µm from the ligation site (*). (B) Imaris (Bitplane) 3D rendered surfaces within area of measurement for α-tubulin (blue), JIP3 (green), DCTN1 (red), and TrkB (cyan).

Results

TrkB results

As TrkB-neurotrophin signaling is implicated in the formation of axonal transport complexes, the expression of TrkB, DCTN1, and JIP3 was also evaluated in conjunction with α-tubulin, an internal control, following sciatic nerve ligation in 120d mice. Protein
accumulation was measured on both sides of the ligation site, in the proximal portion to assess anterograde transport, and in the distal section to evaluate retrograde transport.

Evaluation of proximal α-tubulin with one-way ANOVA revealed that means were not significantly different (p = 0.1892, df = 2, 20, F = 1.829). Bonferroni’s procedure indicated no significant differences in proximal α-tubulin immunofluorescence between groups (Figure 33A): Muscle$^{BDNF+/+}$ versus Muscle$^{BDNF+/-}$ (t = 0.5986, p > 0.05), Muscle$^{BDNF+/+}$ versus Muscle$^{BDNF-/-}$ (t = 1.274, p > 0.05), and Muscle$^{BDNF+/-}$ versus Muscle$^{BDNF-/-}$ (t = 1.873, p > 0.05). One-way ANOVA of distal α-tubulin again showed that means were not significantly different (p = 0.1237, df = 2, 20, F = 2.352). Once more, Bonferroni’s procedure indicated no significant differences in distal α-tubulin immunofluorescence between groups (Figure 33B): Muscle$^{BDNF+/+}$ versus Muscle$^{BDNF+/-}$ (t = 0.8608, p > 0.05), Muscle$^{BDNF+/+}$ versus Muscle$^{BDNF-/-}$ (t = 1.294, p > 0.05), and Muscle$^{BDNF+/-}$ versus Muscle$^{BDNF-/-}$ (t = 2.154, p > 0.05). No significant differences in proximal and distal α-tubulin expression existed between Muscle$^{BDNF+/+}$, Muscle$^{BDNF+/-}$, or Muscle$^{BDNF-/-}$ mice.

Figure 33. Mean surface area (µm$^2$) of α-tubulin immunofluorescence on the proximal (A) and distal (B) sides of ligation in 120d mice. There were no significant differences in α-tubulin immunofluorescence between groups for the proximal (A) and distal (B) ligation sites.
Analysis of proximal TrkB accumulation via one-way ANOVA indicated that means were significantly different (p = 0.0012, df = 2, 20, F = 10.04). Bonferroni’s procedure displayed significant differences in proximal TrkB immunofluorescence between homozygous knockouts compared to both controls and heterozygous knockouts (Figure 34): Muscle$^{BDNF^{+/+}}$ versus Muscle$^{BDNF^{-/-}}$ (t = 4.232, p < 0.01) and Muscle$^{BDNF^{+/+}}$ versus Muscle$^{BDNF^{-/-}}$ (t = 3.394, p < 0.01). There was no significant difference between controls and heterozygous knockouts (Figure 34): Muscle$^{BDNF^{+/+}}$ versus Muscle$^{BDNF^{+/+}}$ (t = 0.8381, p > 0.05). Thus, proximal TrkB immunofluorescence was equivalent in Muscle$^{BDNF^{+/+}}$ and Muscle$^{BDNF^{+/+}}$ animals, but Muscle$^{BDNF^{-/-}}$ mice exhibited significantly reduced TrkB compared to the other genotypes (Figure 34).

Evaluation of distal TrkB accumulation via one-way ANOVA revealed that means were significantly different (p < 0.0001, df = 2, 20, F = 22.04). Bonferroni’s procedure showed no significant difference in distal TrkB immunofluorescence between controls and heterozygous knockouts (Figure 35): Muscle$^{BDNF^{+/+}}$ versus Muscle$^{BDNF^{-/-}}$ (t = 2.600, p > 0.05). Whereas, again, there were significant differences between homozygous knockouts compared to both controls and heterozygous knockouts (Figure 35): Muscle$^{BDNF^{+/+}}$ versus Muscle$^{BDNF^{-/-}}$ (t = 6.591, p < 0.001) and Muscle$^{BDNF^{+/+}}$ versus Muscle$^{BDNF^{-/-}}$ (t = 3.990, p < 0.01). Distally, TrkB appeared to exhibit a trending decline in correlation with reductions in muscle-derived BDNF, however, Muscle$^{BDNF^{-/-}}$ mice were the only group to exhibit statistically significant reduced TrkB accumulation compared to both controls and heterozygous knockouts (Figure 35).
Figure 34. Mean surface area (µm²) of TrkB immunofluorescence accumulation at proximal sciatic nerve ligation site in 120d mice. TrkB immunofluorescence was significantly reduced in Muscle^{BDNF−/−} mice compared to Muscle^{BDNF+/−} and WT animals (n=7; ** = p<0.01). There was no significant difference in TrkB expression between Muscle^{BDNF+/−} and WT mice.

Figure 35. Mean surface area (µm²) of TrkB immunofluorescence accumulation at distal sciatic nerve ligation site in 120d mice. TrkB immunofluorescence was significantly reduced in Muscle^{BDNF−/−} mice compared to Muscle^{BDNF+/−} and Muscle^{BDNF+/+} animals (n=7; ** = p<0.01; *** = p<0.001). There was no significant difference in TrkB expression between Muscle^{BDNF+/−} and WT mice.
Proximal accumulation of the adaptor protein JIP3 was assessed with one-way ANOVA, which revealed that means were significantly different (p = 0.0003, df = 2, 20, F = 13.53). Bonferroni’s procedure indicated significant differences between both heterozygous and homozygous knockouts compared to controls (Figure 36): Muscle$^{BDNF^{+/+}}$ versus Muscle$^{BDNF^{+/-}}$ (t = 3.086, p < 0.05) and Muscle$^{BDNF^{+/+}}$ versus Muscle$^{BDNF^{-/-}}$ (t = 5.169, p < 0.001). Whereas there was no significant difference between knockout groups (Figure 36): Muscle$^{BDNF^{+/-}}$ versus Muscle$^{BDNF^{-/-}}$ (t = 2.083, p > 0.05).

Thus, proximally, JIP3 was significantly reduced in Muscle$^{BDNF^{+/-}}$ and Muscle$^{BDNF^{-/-}}$ animals compared to control mice (Figure 36).

Analysis of distal accumulation of JIP3 with one-way ANOVA indicated that means were significantly different (p < 0.0001, df = 2, 20, F = 21.46). Once again, Bonferroni’s procedure demonstrated that both knockout groups were significantly different compared to controls (Figure 37): Muscle$^{BDNF^{+/+}}$ versus Muscle$^{BDNF^{+/-}}$ (t = 4.707, p < 0.001) and Muscle$^{BDNF^{+/+}}$ versus Muscle$^{BDNF^{-/-}}$ (t = 6.300, p < 0.001). Again, there was no significant difference between knockout groups (Figure 37): Muscle$^{BDNF^{+/-}}$ versus Muscle$^{BDNF^{-/-}}$ (t = 1.593, p > 0.05). Similar to the proximal results, JIP3 accumulation in the distal portion was significantly decreased in Muscle$^{BDNF^{+/-}}$ and Muscle$^{BDNF^{-/-}}$ animals compared to control WT mice (Figure 37).
Figure 36. Mean surface area (µm²) of JIP3 immunofluorescence accumulation at proximal sciatic nerve ligation site in 120d mice. JIP3 immunofluorescence was significantly reduced in Muscle^{BDNF+/-} and Muscle^{BDNF-/-} mice compared to Muscle^{BDNF+/+} animals (n=7; * = p<0.05; *** = p<0.001). There was no significant difference in JIP3 expression between Muscle^{BDNF+/-} and Muscle^{BDNF-/-} mice.

Figure 37. Mean surface area (µm²) of JIP3 immunofluorescence accumulation at distal sciatic nerve ligation site in 120d mice. JIP3 immunofluorescence was significantly reduced in Muscle^{BDNF+/-} and Muscle^{BDNF-/-} mice compared to Muscle^{BDNF+/+} animals (n=7; *** = p<0.001). There was no significant difference in JIP3 expression between Muscle^{BDNF+/-} and Muscle^{BDNF-/-} mice.
One-way ANOVA evaluation of proximal DCTN1 accumulation indicated that means were significantly different ($p = 0.0120$, $df = 2, 20$, $F = 5.715$). Bonferroni’s procedure displayed no significant differences in heterozygous knockouts compared to controls or between knockout groups (Figure 38): Muscles$^{BDNF+/+}$ versus Muscles$^{BDNF+/+}$ ($t = 1.427$, $p > 0.05$) and Muscles$^{BDNF+/-}$ versus Muscles$^{BDNF+/-}$ ($t = 1.941$, $p > 0.05$). Only homozygous knockouts compared to controls exhibited significant changes (Figure 38): Muscles$^{BDNF+/+}$ versus Muscles$^{BDNF+/-}$ ($t = 3.368$, $p < 0.05$). Proximal DCTN1 immunofluorescence indicated a trending decline in BDNF-deficient mice; however, only Muscles$^{BDNF+/-}$ animals exhibited a statistically significant reduction in DCTN1 compared to control WT mice (Figure 38).

Distally, one-way ANOVA analysis of DCTN1 accumulation revealed that, again, means were significantly different ($p < 0.0001$, $df = 2, 20$, $F = 33.69$). Once more, Bonferroni’s procedure indicated that both knockout groups were significantly different compared to controls (Figure 39): Muscles$^{BDNF+/+}$ versus Muscles$^{BDNF+/+}$ ($t = 5.039$, $p < 0.001$) and Muscles$^{BDNF+/-}$ versus Muscles$^{BDNF+/-}$ ($t = 8.131$, $p < 0.001$). However, distally, there was a significant difference in DCTN1 accumulation between knockout groups (Figure 39): Muscles$^{BDNF+/-}$ versus Muscles$^{BDNF+/-}$ ($t = 3.092$, $p < 0.05$). Thus, both Muscles$^{BDNF+/-}$ and Muscles$^{BDNF+/-}$ animals had dramatically reduced DCTN1 accumulation compared to controls, and, additionally, DCTN1 accumulation was significantly reduced in Muscles$^{BDNF+/-}$ animals compared to Muscles$^{BDNF+/+}$ mice (Figure 39).
Figure 38. Mean surface area ($\mu m^2$) of DCTN immunofluorescence accumulation at proximal sciatic nerve ligation site in 120d mice. DCTN1 immunofluorescence was significantly reduced in Muscle$^{BDNF+/\text{ }}$ mice compared to Muscle$^{BDNF+/+}$ animals (n=7; $^* = p<0.05$). There were no significant differences in DCTN1 expression between Muscle$^{BDNF+/+}$ and Muscle$^{BDNF+/-}$ animals, or Muscle$^{BDNF+/-}$ and Muscle$^{BDNF-/-}$ mice.

Figure 39. Mean surface area ($\mu m^2$) of DCTN immunofluorescence accumulation at distal sciatic nerve ligation site in 120d mice. DCTN1 immunofluorescence was significantly reduced in Muscle$^{BDNF+/-}$ and Muscle$^{BDNF-/-}$ mice compared to Muscle$^{BDNF+/+}$ animals (n=7; $^{***} = p<0.001$). DCTN1 expression was also significantly reduced in Muscle$^{BDNF-/-}$ mice compared to Muscle$^{BDNF+/+}$ animals ($^* = p<0.05$).
**p-TrkB results**

To further investigate the potential retrograde transport disruptions in Muscle\textsuperscript{BDNF+/+} and Muscle\textsuperscript{BDNF−/−} mice, and help characterize the role of TrkB-BDNF signaling in axonal transport, the expression of p-TrkB, JIP3, and DCTN1 were assessed along with α-tubulin in ligated sciatic nerves of 120d animals.

Analysis of proximal α-tubulin with one-way ANOVA revealed that means were not significantly different (p = 0.5313, df = 2, 20, F = 0.6552). Bonferroni’s procedure indicated no significant differences in proximal α-tubulin immunofluorescence between groups (Figure 40A): Muscle\textsuperscript{BDNF+/+} versus Muscle\textsuperscript{BDNF+/-} (t = 0.9901, p > 0.05), Muscle\textsuperscript{BDNF+/+} versus Muscle\textsuperscript{BDNF−/−} (t = 0.002612, p > 0.05), and Muscle\textsuperscript{BDNF+/-} versus Muscle\textsuperscript{BDNF−/−} (t = 0.9927, p > 0.05). Evaluation of distal α-tubulin with one-way ANOVA showed that means were not significantly different (p = 0.2933, df = 2, 20, F = 1.314). Bonferroni’s procedure indicated no significant differences in distal α-tubulin immunofluorescence between groups (Figure 40B): Muscle\textsuperscript{BDNF+/+} versus Muscle\textsuperscript{BDNF+/-} (t = 1.552, p > 0.05), Muscle\textsuperscript{BDNF+/+} versus Muscle\textsuperscript{BDNF−/−} (t = 0.3704, p > 0.05), and Muscle\textsuperscript{BDNF+/-} versus Muscle\textsuperscript{BDNF−/−} (t = 1.182, p > 0.05). On both the proximal and distal portions of ligation, there were no significant differences in α-tubulin immunofluorescence between groups (Figures 40A and 40B, respectively). Mean surface area values for α-tubulin were very similar among both TrkB and p-TrkB IHC experiments (Figures 33 and 40).
Analysis of proximal p-TrkB accumulation via one-way ANOVA indicated that means were significantly different (p < 0.0001, df = 2, 20, F = 22.39). Bonferroni’s procedure displayed significant differences in proximal p-TrkB immunofluorescence between all groups (Figure 41): Muscle^{BDNF+/+} versus Muscle^{BDNF+-} (t = 3.232, p < 0.05), Muscle^{BDNF+/+} versus Muscle^{BDNF-/-} (t = 6.691, p < 0.001), and Muscle^{BDNF+-} versus Muscle^{BDNF-/-} (t = 3.459, p < 0.01). Thus, mean surface area of proximal p-TrkB immunofluorescence was significantly reduced in Muscle^{BDNF+-} animals compared to controls, and Muscle^{BDNF-/-} mice exhibited significantly decreased p-TrkB compared to both Muscle^{BDNF+/+} and Muscle^{BDNF+-} animals (Figure 41).

Evaluation of distal p-TrkB accumulation via one-way ANOVA revealed that once more, means were significantly different (p < 0.0001, df = 2, 20, F = 34.18). Again, Bonferroni’s procedure indicated significant differences in distal p-TrkB immunofluorescence between all groups (Figure 42): Muscle^{BDNF+/+} versus Muscle^{BDNF+-} (t = 3.866, p < 0.01), Muscle^{BDNF+/+} versus Muscle^{BDNF-/-} (t = 8.262, p < 0.001), and
Muscle$^{BDNF^+/-}$ versus Muscle$^{BDNF^-/-}$ (t = 4.396, p < 0.01). Distal p-TrkB accumulation was significantly reduced in Muscle$^{BDNF^+/-}$ mice compared to controls, and significantly decreased in Muscle$^{BDNF^-/-}$ animals compared to both control and Muscle$^{BDNF^+/-}$ mice (Figure 42). Altogether, p-TrkB accumulation was statistically different both proximally and distally among all animal groups (Figures 41 and 42).

**Figure 41.** Mean surface area ($\mu$m$^2$) of p-TrkB immunofluorescence accumulation at proximal sciatic nerve ligation site in 120d mice. p-TrkB immunofluorescence was significantly reduced in Muscle$^{BDNF^-/-}$ mice compared to Muscle$^{BDNF^+/-}$ and WT animals (n=7, ** = p<0.01; *** = p<0.001). p-TrkB expression was also significantly diminished in Muscle$^{BDNF^+/-}$ animals compared to Muscle$^{BDNF^+/-}$ mice (* = p<0.05).
Figure 42. Mean surface area (μm²) of p-TrkB immunofluorescence accumulation at distal sciatic nerve ligation site in 120d mice. p-TrkB immunofluorescence was significantly reduced in Muscle^{BDNF+/-} and Muscle^{BDNF-/-} mice compared to WT animals (n=7; ** = p<0.01; *** = p<0.001). p-TrkB expression was also significantly decreased in Muscle^{BDNF-/-} animals compared to Muscle^{BDNF+/-} mice.

Proximal accumulation of JIP3 was assessed with one-way ANOVA, which showed that means were significantly different (p = 0.0022, df = 2, 20, F = 8.776). However, Bonferroni’s procedure indicated significant differences only in homozygous knockouts compared to controls (Figure 43): Muscle^{BDNF+/-} versus Muscle^{BDNF-/-} (t = 4.171, p < 0.01). Whereas there were no significant differences in the heterozygous knockouts compared to controls, or between knockout groups (Figure 43): Muscle^{BDNF+/-} versus Muscle^{BDNF+/-} (t = 1.741, p > 0.05) and Muscle^{BDNF+/-} versus Muscle^{BDNF-/-} (t = 2.430, p > 0.05). Proximally, JIP3 was significantly reduced only in Muscle^{BDNF-/-} animals compared to control mice, but there appeared to be a trending decline in JIP3 expression that correlated with decreases in muscle-derived BDNF (Figure 43).

Analysis of distal JIP3 accumulation using one-way ANOVA showed once more that means were significantly different (p = 0.0002, df = 2, 20, F = 14.22). Again,
Bonferroni’s procedure demonstrated that both knockout groups were significantly different compared to controls (Figure 44): Muscle\textsuperscript{BDNF+/+} versus Muscle\textsuperscript{BDNF+/−} (t = 3.391, p < 0.01) and Muscle\textsuperscript{BDNF+/+} versus Muscle\textsuperscript{BDNF−/−} (t = 5.260, p < 0.001). Again, there was no significant difference between knockout groups (Figure 44): Muscle\textsuperscript{BDNF+/−} versus Muscle\textsuperscript{BDNF−/−} (t = 1.869, p > 0.05). Similar to the JIP3 results in the TrkB IHC experiment (Figures 36 and 37), both Muscle\textsuperscript{BDNF+/−} and Muscle\textsuperscript{BDNF−/−} mice displayed significantly reduced JIP3 immunofluorescence compared to controls (Figure 44).

Figure 43. Mean surface area (µm\textsuperscript{2}) of JIP3 immunofluorescence accumulation at proximal sciatic nerve ligation site in 120d mice. JIP3 immunofluorescence was significantly reduced in Muscle\textsuperscript{BDNF−/−} mice compared to Muscle\textsuperscript{BDNF+/+} animals (n=7; ** = p<0.01). There were no significant differences in JIP3 expression between Muscle\textsuperscript{BDNF+/+} and Muscle\textsuperscript{BDNF+/−} mice, or Muscle\textsuperscript{BDNF+/+} and Muscle\textsuperscript{BDNF−/−} animals.
Figure 44. Mean surface area (µm²) of JIP3 immunofluorescence accumulation at distal sciatic nerve ligation site in 120d mice. JIP3 immunofluorescence was significantly reduced in Muscle^{BDNF+/+} and Muscle^{BDNF-/-} mice compared to Muscle^{BDNF+/+} animals (n=7, ** = p<0.01; *** = p<0.001). There was no significant difference in JIP3 expression between Muscle^{BDNF+/+} and Muscle^{BDNF-/-} mice.

Evaluation of proximal DCTN1 accumulation using one-way ANOVA showed that means were significantly different (p = 0.0024, df = 2, 20, F = 8.557). However, Bonferroni’s procedure indicated that proximal DCTN1 accumulation was significantly reduced only in homozygous knockouts compared to both control and heterozygous knockout animals (Figure 45): Muscle^{BDNF+/+} versus Muscle^{BDNF-/-} (t = 3.984, p < 0.01) and Muscle^{BDNF+/+} versus Muscle^{BDNF-/-} (t = 2.957, p < 0.05). Whereas once more, there was no difference between control and heterozygous mice (Figure 45): Muscle^{BDNF+/+} versus Muscle^{BDNF+/+} (t = 1.027, p > 0.05). Proximally, DCTN1 accumulation was significantly reduced in Muscle^{BDNF+/+} animals compared to both controls and heterozygous knockouts (Figure 45).
One-way ANOVA assessment of distal DCTN1 accumulation again showed that means were significantly different (p < 0.0001, df = 2, 20, F = 20.39). Similar to the results from the TrkB IHC experiment, Bonferroni’s procedure indicated that DCTN1 immunofluorescence was significantly different between all groups (Figure 46):

Muscle$^{BDNF+/+}$ versus Muscle$^{BDNF+/-}$ ($t = 2.895$, $p < 0.05$), Muscle$^{BDNF+/+}$ versus Muscle$^{BDNF-/-}$ ($t = 6.376$, $p < 0.001$), and Muscle$^{BDNF+/-}$ versus Muscle$^{BDNF-/-}$ ($t = 3.482$, $p < 0.01$). Thus, nearly identical to the TrkB IHC experiment results (Figure 39), distal DCTN1 accumulation was significantly reduced in both Muscle$^{BDNF+/-}$ and Muscle$^{BDNF-/-}$ animals compared to controls, and significantly decreased in Muscle$^{BDNF-/-}$ animals compared to Muscle$^{BDNF+/-}$ mice (Figure 46).

Figure 45. Mean surface area ($\mu m^2$) of DCTN1 immunofluorescence accumulation at proximal sciatic nerve ligation site in 120d mice. DCTN1 immunofluorescence was significantly reduced in Muscle$^{BDNF-/-}$ mice compared to Muscle$^{BDNF+/-}$ and Muscle$^{BDNF+/+}$ animals ($n=7$; $* = p<0.05$, $** = p<0.01$). There was no significant difference in DCTN1 expression between Muscle$^{BDNF+/-}$ and Muscle$^{BDNF+/-}$ mice.
Discussion

In order to assess potential disruptions in axonal transport due to reduced or absent muscle-synthesized BDNF, accumulation of transport complex-associated proteins was measured immediately proximally and distally to the sciatic nerve ligation site in 120d transgenic mice. Two separate but very similar experiments were performed to evaluate the expression of α-tubulin, JIP3, and DCTN1 along with TrkB, or its activated form, p-TrkB. These results, in conjunction with prior ligation studies conducted by the Ottem lab, help to better characterize anterograde and retrograde axonal transport in Muscle^{BDNF-/-} deficient mice.

In each experiment, both proximally and distally, the mean surface area of α-tubulin immunofluorescence was not significantly different between animal groups.
(Figures 33 and 40). In fact, the values appeared to be very similar across the board, indicating a high degree of consistency in the IHC assays and Imaris® data collection between the two experiments.

Proximal to the ligation site, TrkB expression was equivalent in Muscle\textsuperscript{BDNF+/+} and Muscle\textsuperscript{BDNF+/−} mice, but significantly reduced in Muscle\textsuperscript{BDNF−/−} animals compared to both groups (Figure 34). These findings are consistent with the previous results for TrkB immunofluorescence in presynaptic terminals of gastroc-associated NMJs (Figure 28). As mentioned earlier, BDNF is transported anterogradely from neuron cell bodies to their terminals and, at 120d of age, heterozygous knockout mice display significantly increased BDNF immunofluorescence at the cell soma of gastroc-associated motor neurons (Figure 8B; Altar et al., 1997; Pomeroy, 2013). BDNF has been shown to promote the anterograde axonal transport and trigger membrane insertion of TrkB, which may explain the equivalent levels of TrkB accumulation on the proximal side of ligation in Muscle\textsuperscript{BDNF+/+} and Muscle\textsuperscript{BDNF+/−} mice (Cheng et al., 2011). BDNF also regulates the surface expression of TrkB, which could explain the equivalent levels in Muscle\textsuperscript{BDNF+/+} and Muscle\textsuperscript{BDNF+/−} mice, and the reduced levels in Muscle\textsuperscript{BDNF−/−} animals (Haapasalo et al., 2002). Conversely, the \textit{BDNF} gene has multiple promoters and differential splicing patterns which control the expression and trafficking of BDNF, so an increase of BDNF at the cell soma does not necessarily mean increased anterograde transport of BDNF (Timmusk et al., 1993; Aid et al., 2007). Additionally, JIP3 is significantly reduced in Muscle\textsuperscript{BDNF+/−} and Muscle\textsuperscript{BDNF−/−} mice compared to controls (Figure 36). Because this adaptor protein is essential for the anterograde transport of TrkB, it is somewhat surprising to see a reduction in TrkB accumulation on the proximal side of ligation only
in homozygous knockout mice (Huang et al., 2011; Sun et al., 2017). A previous study found that TrkB expression is upregulated in the spinal cord following sciatic nerve ligation, which could account for the increased accumulation of TrkB in Muscle^{BDNF+/−} mice (Narita et al., 2000). However, in the experiment by Narita and colleagues, the sciatic nerve was ligated for 14d, while in the present study, the sciatic nerve was ligated for only 8 hours. Thus, it is unlikely that any significant changes in TrkB expression in the spinal cord happened during this time period (Narita et al., 2000). Additionally, any changes in TrkB expression would have occurred in all genotypes, and therefore would be negligible.

In the distal portion of ligated sciatic nerve, TrkB immunofluorescence was significantly reduced in Muscle^{BDNF−/−} animals compared to Muscle^{BDNF+/−} and Muscle^{BDNF+/−} mice (Figure 35). While the levels of TrkB accumulation were not statistically different, TrkB did appear to be slightly lower in Muscle^{BDNF+/−} mice compared to controls. These data do not deviate from previous results, as the levels of TrkB were found to be roughly equivalent in the presynaptic terminals of gastroc-associated motor neurons in Muscle^{BDNF+/−} and Muscle^{BDNF+/−} mice. In addition, endosomal transport of TrkB by dynein-dynactin complexes from an equivalent starting pool should elicit a consistent level of protein accumulation as long as transport mechanisms are intact (Figure 28). Previous findings indicated disrupted retrograde transport only in Muscle^{BDNF−/−} animals, which, combined with the decreased levels of TrkB in presynaptic terminals of gastroc-associated NMJs, likely accounts for reduced accumulation of the receptor on the distal side of ligation in these mice (Figures 14 and 15; Dangremond, 2016).
Assessment of p-TrkB accumulation on the proximal side of ligation revealed significant reductions in Muscle^{BDNF+/-} and Muscle^{BDNF-/-} mice compared to controls, and in Muscle^{BDNF-/-} animals compared to Muscle^{BDNF+/+} mice (Figure 41). In theory, with equivalent amounts of TrkB transported anterogradely in Muscle^{BDNF+/+} and Muscle^{BDNF+/+} mice, combined with increased BDNF expression at the cell soma in Muscle^{BDNF+/+} animals, comparable levels of p-TrkB accumulation were expected between WT animals and heterozygous knockouts (Figures 8 and 34; Pomeroy, 2013). However, studies using neuroblastoma cells and primary hippocampal neurons have demonstrated that BDNF differentially regulates the surface expression of TrkB based on treatment times, which may have had an influence on the levels of p-TrkB accumulation (Haapasalo et al., 2002). Additionally, as previously mentioned, the BDNF gene has multiple promoters and splicing patterns which direct the expression and trafficking of BDNF (Timmusk et al., 1993; Aid et al., 2007). Thus, the increased BDNF immunofluorescence observed in the cell soma of heterozygous knockouts may have no effect on the activation and transport of TrkB, leading to significantly decreased levels of p-TrkB in these mice.

On the distal side of the ligation site, p-TrkB accumulation was significantly reduced in Muscle^{BDNF+/-} mice compared to controls, and Muscle^{BDNF-/-} animals compared to both WT and heterozygous knockout mice (Figure 42). These results could be explained by a number or combination of factors. The most apparent being, a lack – or absence – of BDNF in muscle leads to reduced TrkB receptor activation in the presynaptic terminal which leads to a decrease in p-TrkB accumulation at the distal ligation site, as BDNF stimulation has been shown to be required for the initiation of
retrograde transport of TrkB (Heerssen et al., 2004; Mitchell et al., 2012). Another plausible cause may be reduced JIP3 expression observed in Muscle\textsuperscript{BDNF+/−} and Muscle\textsuperscript{BDNF−/−} mice, as JIP3 has been shown to be essential for BDNF-induced retrograde signaling of TrkB (Huang et al., 2011; Sun et al., 2017). One other potential source for the reductions observed in Muscle\textsuperscript{BDNF+/−} and Muscle\textsuperscript{BDNF−/−} mice may be due to impairments in retrograde transport, as discussed below.

Proximally, JIP3 accumulation was significantly reduced in both Muscle\textsuperscript{BDNF+/−} and Muscle\textsuperscript{BDNF−/−} mice compared to controls in the TrkB IHC (Figure 36) and also significantly diminished in Muscle\textsuperscript{BDNF−/−} mice in the p-TrkB IHC (Figure 43). The anterograde axonal delivery of TrkB is mediated by kinesin-1, which directly associates with TrkB via interaction with JIP3 (Huang et al., 2011; Sun et al., 2017). The binding of JIP3 to kinesin-1 is essential for TrkB axonal transport in the anterograde direction (Sun et al., 2017). Thus, observed decreases in JIP3 accumulation on the proximal side may account for the reduced anterograde transport of TrkB in Muscle\textsuperscript{BDNF−/−} mice (Figure 34). These data suggest impairments in anterograde axonal transport or in the assembly of anterograde transport complexes in Muscle\textsuperscript{BDNF} deficient animals.

On the distal side of the ligation site, JIP3 accumulation was significantly decreased in Muscle\textsuperscript{BDNF+/−} and Muscle\textsuperscript{BDNF−/−} mice compared to controls in both the TrkB and p-TrkB IHC experiments (Figures 37 and 44, respectively). Given that JIP3 has been shown to be a vital adaptor protein for axonal transport and essential for BDNF-induced TrkB retrograde signaling, these results verify the critical importance of muscle-synthesized BDNF for the initiation of retrograde transport and assembly of transport complexes (Huang et al., 2011; Sun et al., 2017). The impairments in JIP3’s ability to
help bind retrograde transport complexes to motor proteins due to diminished or lack of muscle-derived BDNF may account for the decreased accumulation of TrkB and p-TrkB, as mentioned previously, and DCTN1, discussed below, in Muscle\textsuperscript{BDNF} deficient mice.

On the proximal side of the ligation site, DCTN1 accumulation was significantly reduced in Muscle\textsuperscript{BDNF-/} mice compared to controls in the TrkB IHC (Figure 38). In the p-TrkB IHC, DCTN1 was significantly decreased in Muscle\textsuperscript{BDNF-/} animals when compared to both heterozygous knockouts and controls (Figure 45). These results are in conflict with the findings from Dangremond, 2016, where DCTN1 accumulation was significantly increased in Muscle\textsuperscript{BDNF+/-} and Muscle\textsuperscript{BDNF-/} mice when compared to control animals (Figure 18). The outcome of the previous study suggested potentially upregulated anterograde axonal transport in Muscle\textsuperscript{BDNF} knockout mice (Dangremond, 2016). However, the combined results for TrkB, p-TrkB, JIP3, and DCTN1 from the present experiments indicate impairments in anterograde transport due to decreased levels of muscle-derived BDNF, as JIP3 and DCTN1 are integral proteins for the assembly of transport complexes (Huang et al., 2011; Moughamian and Holzbaur, 2012; Moughamian et al., 2013; Sun et al., 2017). The discrepancies between the previous and present results may be due to a number of factors. However, the most plausible explanation is variability in the IHC staining and Imaris\textsuperscript{®} data collection techniques in the previous study, as there was no evaluation of an internal control such as α-tubulin. These variations may have been due to multiple research assistants helping with experimental techniques in the previous study, while all of the staining and data collection in the present study was conducted by a single researcher.
Distally, the accumulation of DCTN1 was significantly reduced in Muscle^{BDNF+/−} and Muscle^{BDNF−/−} mice when compared to controls, and in Muscle^{BDNF−/−} mice compared to Muscle^{BDNF+/−} animals in the TrkB experiment (Figure 39). The results for DCTN1 immunofluorescence on the distal side of ligation were identical in the p-TrkB experiment (Figure 46). These data, in association with the results for TrkB, p-TrkB, and JIP3 accumulation, suggest significant deficiencies in retrograde axonal transport mechanisms and retrograde neurotrophin signaling due to a lack or absence of muscle-synthesized BDNF.

The results for the TrkB and p-TrkB IHC experiments are modeled in Figures 47 and 48 below.
Figure 47. Summary of sciatic nerve ligation protein accumulation results for TrkB IHC experiment. Results for Muscle^{BDNF+/+}, Muscle^{BDNF+/-}, and Muscle^{BDNF-/-} animals are shown in (A), (B), and (C), respectively. Accumulation of anterogradely transported TrkB is represented by the purple filled shape, JIP3 by light green, and DCTN1 by dark green. Accumulation of retrogradely transported TrkB is represented by the red filled shape, JIP3 by orange, and DCTN1 by yellow. Anterograde transport of JIP3 was significantly reduced in heterozygous knockouts (B) compared to controls (A). Retrograde transport of JIP3 and DCTN1 was significantly decreased in heterozygous knockouts (B) compared to controls (A). Anterograde transport of TrkB was significantly reduced in homozygous knockouts (C) compared to controls (A) and heterozygous knockouts (B). Anterograde transport of JIP3 and DCTN1 was significantly reduced in homozygous knockouts (C) compared to controls (A). Retrograde transport of JIP3 was significantly reduced in homozygous knockouts (C) compared to controls (A). Retrograde transport of TrkB and DCTN1 was significantly decreased in homozygous knockouts (C) compared to both controls (A) and heterozygous knockouts (B).
Figure 48. Summary of sciatic nerve ligation protein accumulation results for p-TrkB IHC experiment. Results for Muscle^{BDNF+/+}, Muscle^{BDNF+/−}, and Muscle^{BDNF−/−} animals are shown in (A), (B), and (C), respectively. Accumulation of anterogradely transported p-TrkB is represented by the purple filled shape, JIP3 by light green, and DCTN1 by dark green. Accumulation of retrogradely transported p-TrkB is represented by the red filled shape, JIP3 by orange, and DCTN1 by yellow. Anterograde transport of p-TrkB was significantly reduced in heterozygous knockouts (B) compared to controls (A), while transport of JIP3 was reduced, but not significantly different, in heterozygous knockouts (B) compared to controls (A). Retrograde transport of p-TrkB, JIP3, and DCTN1 was significantly decreased in heterozygous knockouts (B) compared to controls (A). Anterograde and retrograde transport of p-TrkB and DCTN1 was significantly reduced in homozygous knockouts (C) compared to both controls (A) and heterozygous knockouts (B). Anterograde and retrograde transport of JIP3 was significantly decreased in homozygous knockouts (C) compared to controls (A).
Together, the results of the present study indicate the significance of muscle-synthesized BDNF, and BDNF-TrkB signaling, for both anterograde and retrograde axonal transport. The results for the TrkB and p-TrkB IHC experiments are depicted in Figures 47 and 48, respectively. The consistency of α-tubulin immunofluorescence proximally and distally across all genotypes for both experiments indicates uniform IHC staining techniques and Imaris® data collection procedures.

The accumulation of every labeled protein was significantly reduced in Muscle<sup>BDNF</sup>-/ mice compared to control animals both proximally and distally. Moreover, proximally and distally, TrkB and p-TrkB accumulation was significantly reduced in Muscle<sup>BDNF</sup>-/ mice compared to Muscle<sup>BDNF</sup>+/- animals. In the TrkB experiment, DCTN1 was significantly reduced in Muscle<sup>BDNF</sup>-/ animals compared to Muscle<sup>BDNF</sup>+/- mice on the distal side of ligation, but not proximally. On the other hand, DCTN1 accumulation in the p-TrkB experiment was significantly reduced in Muscle<sup>BDNF</sup>-/ mice compared to Muscle<sup>BDNF</sup>+/- animals both proximally and distally. The lack of significant differences in JIP3 accumulation between Muscle<sup>BDNF</sup>+/- and Muscle<sup>BDNF</sup>-/ animals suggest that a certain level or threshold of muscle-synthesized BDNF is required to ensure the proper function of JIP3, because similar levels of JIP3 accumulation were observed with reduced and absent muscle-derived BDNF. The other proteins, TrkB, p-TrkB, and DCTN1, do not display the same threshold requirement, exhibiting significantly different levels of accumulation between Muscle<sup>BDNF</sup>+/- and Muscle<sup>BDNF</sup>-/ mice.

Evaluation of protein accumulation in Muscle<sup>BDNF</sup>+/- animals revealed a similar, but slightly different story. Both proximally and distally, TrkB accumulation was equivalent with controls animals, while p-TrkB accumulation was significantly reduced
compared to controls. In the TrkB experiment, JIP3 accumulation was significantly reduced in Muscle\textsuperscript{BDNF+/-} animals compared to controls on both the proximal and distal sides of ligation. Alternatively, JIP3 accumulation in the p-TrkB experiment was significantly reduced in Muscle\textsuperscript{BDNF+/-} mice compared to controls distally, but not proximally. In both IHC experiments, DCTN1 accumulation on the proximal side of ligation was not significantly different from control animals, while distally, DCTN1 accumulation was significantly reduced in Muscle\textsuperscript{BDNF+/-} animals compared to controls. The slightly differing results between the two IHC experiments suggest that while axonal transport mechanisms are attempting to function normally, the reduced levels of muscle-derived BDNF in these animals are not adequate to properly drive anterograde and retrograde transport. Additionally, retrograde transport appears to be more severely impaired than anterograde transport in Muscle\textsuperscript{BDNF+/-} animals. This is evidenced by the far fewer instances of significant changes from control animals on the proximal side of ligation. On the proximal side, only two out of the six proteins assessed were significantly different in Muscle\textsuperscript{BDNF+/-} mice compared to control animals, while distally, five out of six were significantly different compared to controls.

These data suggest significant impairments in anterograde and retrograde axonal transport due to a lack of muscle-derived BDNF and the importance of BDNF-TrkB signaling in these transport mechanisms. Reduced accumulation of protein on the proximal side of ligation indicates that anterograde transport mechanisms are impaired and that TrkB activation and signaling pathways are significantly reduced at the cell body. Additionally, TrkB activation and signaling is also inhibited at the distal axon, evidenced by the dysfunctional retrograde transport and decreased accumulation of
protein on the distal side of ligation. Furthermore, JIP3 appears to exhibit a threshold requirement for the amount of BDNF needed to ensure proper functionality, as reduced and absent muscle-synthesized BDNF elicit equivalent levels of JIP3 accumulation. These results also indicate that perhaps ligation of the sciatic nerve was not particularly needed, and protein assays could have been used instead to determine expression levels of α-tubulin, TrkB, p-TrkB, JIP3, and DCTN1 in experimental animals. However, the ligation was important because it revealed that retrograde transport appears to be more severely impaired than anterograde transport in Muscle\textsuperscript{BDNF+/−} mice. Future studies should further characterize the apparent axonal transport defects in mice missing muscle-synthesized BDNF by assessing BDNF-TrkB downstream signaling cascades at the cell soma and NMJ. It may be beneficial to compare mRNA levels of TrkB, JIP3, and DCTN in motor neurons of Muscle\textsuperscript{BDNF} deficient mice at varying stages of development. This would help determine whether transport defects are occurring because of decreased synthesis of essential transport complex proteins, or if there are post-translational mechanisms leading to reductions in axonal transport. Additionally, it would be of interest to evaluate other critical adaptor proteins to determine if axonal transport deficits are due to disruptions in the assembly of transport complexes.
CHAPTER FIVE: SUMMARY AND CONCLUSIONS

Axonal transport is the process by which cellular components are packaged into vesicles and transported by motor proteins either from the cell soma to the distal dendrites, anterogradely, or in the retrograde direction, from the dendrites back to the soma. Defects in axonal transport, especially retrograde, have been implicated in a number of neuromuscular diseases such as SBMA, HD, and ALS (Goldstein and Yang, 2000; Morfini et al., 2009). BDNF-TrkB signaling, DCTN1, and JIP3 have been shown to be important for the initiation of axonal transport (Huang et al., 2011; Mitchell et al., 2012; Moughamian et al., 2013; McKenney et al., 2014; Sun et al., 2017).

The Ottem laboratory has utilized the Cre/lox system to generate and study mice missing muscle-synthesized BDNF. Previous studies labeled motor neuron dendrites using Fluoro-Gold™, a commercially available retrograde tracer molecule, and found that dendritic length was diminished in Muscle^{BDNF-/-} deficient mice (Pomeroy, 2013). These results suggested potential retrograde axonal transport dysfunction in mice missing muscle-derived BDNF and were further investigated using sciatic nerve ligation studies. In 120d animals, this study reported significantly impaired retrograde transport only in Muscle^{BDNF-/-} mice, but intact anterograde transport mechanisms in Muscle^{BDNF+/-} and Muscle^{BDNF-/-} animals (Dangremond, 2016).

The present study has expanded on these findings to further characterize the apparent retrograde transport defects in mice missing muscle-synthesized BDNF and determine the role of muscle-derived BDNF-TrkB signaling in axonal transport. First, the expression of TrkB and its activated form, p-TrkB, were assessed in
the pre- and postsynaptic terminals of gastroc-associated NMJs of 120d mice. In the presynapse, TrkB expression appeared lower in Muscle^{BDNF-/-} animals, but was not significantly different among genotypes (Figure 28). Postsynaptic TrkB, however, was significantly reduced in both Muscle^{BDNF+/-} and Muscle^{BDNF-/-} animals compared to controls (Figure 29). Similarly, both pre- and postsynaptic p-TrkB expression was significantly decreased in Muscle^{BDNF+/-} and Muscle^{BDNF-/-} mice compared to control animals (Figures 30 and 31, respectively). These results indicate that presynaptic expression of TrkB is not significantly impacted by reduced or absent muscle-synthesized BDNF, whereas postsynaptic TrkB expression is significantly decreased. Additionally, both pre- and postsynaptic activation of TrkB is significantly impaired due to reduced or absent muscle-synthesized BDNF, as evidenced by significantly reduced pre- and postsynaptic p-TrkB expression in heterozygous and homozygous knockout animals. Together, these data suggest severe impairments in both pre- and postsynaptic BDNF-TrkB signaling at the NMJ due to reduced or absent muscle-synthesized BDNF.

Additionally, this study employed sciatic nerve ligation experiments to further assess and characterize axonal transport deficits in mice missing muscle-synthesized BDNF. In each experiment, both proximally and distally, the mean surface area of α-tubulin immunofluorescence was nearly identical across animal groups, suggesting a high level of consistency throughout the IHC staining and Imaris® data collection procedures (Figures 33 and 40, respectively).

Measuring protein accumulation on the proximal side of ligation allowed for the evaluation of axonal transport mechanisms in the anterograde direction. TrkB accumulation was significantly reduced in Muscle^{BDNF-/-} animals compared to
Muscle$^{BDNF+/-}$ and control mice on the proximal side of ligation (Figure 34). Proximally, p-TrkB accumulation was significantly decreased in Muscle$^{BDNF+/-}$ and Muscle$^{BDNF-/-}$ animals compared to controls, and in Muscle$^{BDNF-/-}$ mice compared to Muscle$^{BDNF+/-}$ animals (Figure 41). JIP3 immunofluorescence was significantly reduced in Muscle$^{BDNF+/-}$ and Muscle$^{BDNF-/-}$ animals compared to controls in the TrkB IHC (Figure 36), significantly diminished in Muscle$^{BDNF-/-}$ mice compared to controls and reduced, but not significantly, in Muscle$^{BDNF+/-}$ animals compared to controls in the p-TrkB IHC (Figure 43). Proximally, DCTN1 accumulation was significantly reduced in Muscle$^{BDNF-/-}$ animals compared to controls in the TrkB IHC, and compared to both Muscle$^{BDNF+/-}$ and control mice in the p-TrkB IHC (Figures 38 and 45, respectively).

The results obtained from evaluating protein accumulation on the proximal side of ligation demonstrate clear impairments in anterograde axonal transport mechanisms in Muscle$^{BDNF-/-}$ animals, and potential disruptions in Muscle$^{BDNF+/-}$ mice. Because the accumulation of every labeled protein was significantly diminished on the proximal side of ligation in Muscle$^{BDNF-/-}$ mice, it is apparent that these animals are exhibiting defective anterograde transport mechanisms. As mentioned previously, BDNF expression is upregulated at the cell soma of gastroc-associated motor neurons in Muscle$^{BDNF+/-}$ animals, and BDNF has been shown to be anterogradely transported as well as induce the anterograde transport and membrane insertion of TrkB (Altar et al., 1997; Cheng et al., 2011; Pomeroy, 2013). Because of this compensatory upregulation of BDNF at the cell soma, it is possible that anterograde transport mechanisms are impaired in Muscle$^{BDNF+/-}$ animals, but are masked by greatly heightened transport of TrkB-containing vesicles from the cell body to the dendrites in these mice.
To assess the efficacy of retrograde axonal transport mechanisms, mean surface area of protein accumulation on the distal side of ligation was measured. TrkB immunofluorescence was significantly diminished in Muscle$^{BDNF-/-}$ animals compared to heterozygous knockout and control mice (Figure 35). Accumulation of p-TrkB was significantly reduced in both Muscle$^{BDNF+/+}$ and Muscle$^{BDNF-/-}$ animals compared to controls, as well as in Muscle$^{BDNF-/-}$ animals compared to Muscle$^{BDNF+/+}$ mice (Figure 42). In both IHC stains, JIP3 immunofluorescence was significantly reduced in Muscle$^{BDNF+/+}$ and Muscle$^{BDNF-/-}$ animals compared to controls (Figures 37 and 44, respectively). Furthermore, DCTN1 accumulation was significantly decreased in Muscle$^{BDNF+/+}$ and Muscle$^{BDNF-/-}$ animals compared to controls, and in Muscle$^{BDNF-/-}$ animals compared to Muscle$^{BDNF+/+}$ mice for the TrkB and p-TrkB experiments (Figures 39 and 46). These data suggest significant dysfunctions in retrograde transport mechanisms in both Muscle$^{BDNF+/+}$ and Muscle$^{BDNF-/-}$ animals.

Together, data from the present studies indicate the importance of BDNF-TrkB signaling for axonal transport as well as significant impairments in anterograde and retrograde transport mechanisms because of reduced or absent muscle-derived BDNF. The results for the TrkB and p-TrkB experiments are depicted in Figures 47 and 48, respectively. Reduced accumulation of protein on the proximal side of ligation indicates that anterograde transport mechanisms are impaired and that TrkB activation and signaling pathways are significantly reduced at the cell body. Furthermore, TrkB activation and signaling is inhibited at the distal axon as well, which is evidenced by the dysfunctional retrograde transport and decreased accumulation of protein on the distal side of ligation. In addition, JIP3 appeared to require a certain threshold of BDNF for
proper functioning. Reduced and absent muscle-derived BDNF caused equivalent accumulation of JIP3 on the proximal and distal sides of ligation, and these levels were significantly reduced compared to controls. Retrograde transport was found to be markedly more impaired compared to anterograde transport in Muscle^{BDNF+/−} animals, however, both transport mechanisms were diminished compared to controls. This suggests that while axonal transport is attempting to proceed normally, the reduced levels of muscle-synthesized BDNF are not adequate to ensure proper functionality of these transport mechanisms. Muscle^{BDNF−/−} mice exhibited major deficiencies in anterograde and retrograde axonal transport compared to controls. Furthermore, accumulation of TrkB, p-TrkB, and DCTN1 were significantly reduced in Muscle^{BDNF−/−} mice compared to Muscle^{BDNF+/−} animals, emphasizing the importance of muscle-synthesized BDNF and indicating a direct correlation between the level of BDNF and the transport of these proteins.

Future studies should investigate the levels of TrkB and p-TrkB expression in gastroc tissue of transgenic mice at varying ages using protein quantification assays. It may be beneficial to also assess the abundance of TrkB mRNA in gastroc tissue of these mice using Northern blotting, in situ hybridization, or reverse transcription PCR techniques. Because of the threshold requirement potentially exhibited by JIP3, it would be interesting to evaluate whether treatment with BDNF restores its functionality in these knockout mice. To further characterize the apparent axonal transport defects, it would be helpful to assess BDNF-TrkB downstream signaling cascades at the cell soma and NMJ. It may be beneficial to compare mRNA levels of TrkB, JIP3, and DCTN in motor neurons of these transgenic mice. This would help define whether transport defects are
occurring because of decreased synthesis of essential transport complex proteins, or if there are post-translational mechanisms leading to reductions in axonal transport. Finally, it may be advantageous to evaluate other critical adaptor proteins and motor proteins to determine if axonal transport deficits are due to disruptions in the assembly or the motility of transport complexes.
REFERENCES


Pomeroy EJ (2013) The Role of Muscle-Synthesized Brain-Derived Neurotrophic Factor (BDNF) in the Health and Maintenance of Motorneurons.


Williamson TL, Cleveland DW (1999) Slowing of axonal transport is a very early event in the toxicity of ALS-linked SOD1 mutants to motor neurons. Nat Neurosci 2:50–56.


APPENDIX A

APPROVAL OF ANIMAL USE BY INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

Application to Use Vertebrate Animals in Research, Testing or Instruction

Application Number: 241
Date Amended
Application Received: 6/6/14
Date of Amendment
Approval: 6/27/14

General Instructions
All parts of this form can be submitted electronically to the Institutional Animal Care and Use Committee (email: IACUC@nmu.edu), EXCEPT for the signature page. Review of this application will commence upon receiving the electronic application, but an application cannot be approved without all required signatures on the hardcopy signature page (send to IACUC/Graduate Education and Research/401 Cohodas Hall). Please contact the IACUC chair (email: IACUCChr@nmu.edu) if you have any questions.

Review Dates:
Designated Member Review of applications (appropriate for USDA Use Categories B and C) will be completed within two weeks after receipt of the electronic application.

Full Committee Review of applications will take place on the third Friday of every month. Applications for Full Committee Review must be electronically received by the first Friday of the month. Applications are reviewed by the full IACUC meeting for USDA Use Categories D and E. A USDA Use Category B or C may be reviewed at a Full IACUC meeting if requested by an IACUC member. Detailed procedures on the IACUC review processes are located at the IACUC website.
I. Principal Investigator (Must be a faculty member or Department Head)

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Co-Investigator
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Phone number
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Date
02/28/2014

II. Project/Grant/Course Number and Title (If you will be using external funds, please use the same title as the grant application; if work is for a course, please include the number of the course, title of the course, and a title for the work proposed)

Investigating axonal retrograde transport in motor neurons of muscle-synthesized BDNF deficient mice

Funding Sources (External & Internal) External: NIH/NINDS R15 AREA Grant

Project/Course Start and End Dates March 2014 to March 2017 (three year maximum)

Additional Funding Pending? □ Yes ☒ No