THE ROLE OF SKELETAL MUSCLE-SYNTHESIZED BRAIN DERIVED NEUROTROPHIC FACTOR IN THE MAINTENANCE OF MOTOR NEURON MITOCHONDRIAL POPULATIONS

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THE ROLE OF SKELETAL MUSCLE-SYNTHESIZED BRAIN DERIVED NEUROTROPHIC FACTOR IN THE MAINTENANCE OF MOTOR NEURON MITOCHONDRIAL POPULATIONS

By

Mikel L. Cawley

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THE ROLE OF SKELETAL MUSCLE-SYNTHESIZED BRAIN DERIVED NEUROTROPHIC FACTOR IN THE MAINTENANCE OF MOTOR NEURON MITOCHONDRIAL POPULATIONS

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ABSTRACT

THE ROLE OF SKELETAL MUSCLE-SYNTHESIZED BRAIN DERIVED NEUROTROPHIC FACTOR IN THE MAINTENANCE OF MOTOR NEURON MITOCHONDRIAL POPULATIONS

By
Mikel L. Cawley

Mitochondria are essential for the high energy demands of the neuromuscular junction and, as a consequence, leave motorneurons susceptible to dysfunction. A potential origin of progressive pathology may be a reduction in brain-derived neurotrophic-factor (BDNF) signaling at the motor unit. We have shown that mice deficient in skeletal muscle-synthesized BDNF (msBDNF) demonstrate progressive motorneuron and muscle pathology at 120d. We hypothesize mitochondrial populations will be altered in motorneurons of msBDNF deficient-mice. At 117d, msBDNF deficient-mice received intramuscular injections of MitoTracker™ dye targeting the right gastrocnemius muscle. At 120d experimental groups underwent a gastrocnemius harvest or a sciatic nerve ligation protocol prior to sacrifice. In combination with MitoTracker™ injections, we used immunohistochemical labeling to target the mitochondrial translocase of outer membrane, TOM20. We used immunolabeling to further delineate mitochondrial populations within gastroc-associated motorneurons. To determine if experimental groups exhibit altered mitochondrial populations, we used confocal microscopy and IMARIS 3D rendering software. Immunolocalization was measured throughout the axon and axon terminals of motorneurons. We found no significant difference in mitochondrial populations within the pre-or post-synapse of control and msBDNF deficient animals. Results indicate that co-labeling mitochondria in a sciatic nerve ligation model illustrate mitochondrial population, as well as utility during transport. We found no significant difference in mitochondrial populations along the motor axon of msBDNF deficient mice at 120d. Ongoing studies suggest that mitochondrial populations and mobility may be as dynamic as mitochondrial function and assisted by the coexistence of several signaling mechanisms.
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LIST OF ABBREVIATIONS

AChR…………………………………… Acetylcholine Receptor

AD…………………………………… Alzheimer’s Disease

ADP…………………………………… Adenosine Diphosphate

Akt…………………………………… Protein Kinase B

ALS…………………………………… Amyotrophic Lateral Sclerosis

ATP…………………………………… Adenosine Triphosphate

BAD…………………………………… BCL2-associated Death Promoter

BCL2…………………………………… B-cell Lymphoma 2

BDNF…………………………………… Brain Derived Neurotrophic Factor

BMECs……………………………… Brain Microvascular Endothelial Cells

BNIP3…………………………………… BCL2 Interacting Protein 3

cAMP…………………………………… cyclic Adenosine Monophosphate

CMT…………………………………… Charcot-Marie- Tooth Disease

CREB…………………………………… cAMP Response Element-Binding Protein

d…………………………………… days old

DAG…………………………………… Diacylglycerol
DCTN1............................. Dynactin Subunit 1

DMSO............................... Dimethyl Sulfoxide

DRP1............................... Dynamin Related Protein 1

EF-hand............................ Helix-Loop-Helix Structural Domain

fALS............................... familial Amyotrophic Lateral Sclerosis

Flox............................... Lox-flanked

GABARAP......................... Gamma-aminobutyric Acid Receptor-associated Protein

HIF-1α............................ Hypoxia-inducible factor 1-alpha

HSA............................... Human Skeletal Actin

IM................................. Intramuscular

IP3................................. Inositol Trisphosphate

LC3................................. Microtubule-associated proteins 1A/1B light chain 3B

MAPK.............................. Mitogen Activated Protein K

MDV............................... Mitochondrial Derived Vesicle

MEK............................... Mitogen Activated Protein Kinase Kinase

MFN1.............................. Mitofusion Protein 1

MFN2.............................. Mitofusion Protein 2

MIRO............................. Mitochondrial Rho GTPase
msBDNF................................. muscle-synthesized BDNF

mtDNA................................. Mitochondrial DNA

MUL1................................. Mitochondrial E3 ubiquitin protein ligase 1

NDS................................. Normal Donkey Serum

NF-H-P................................. Hyperphosphorylated Neurofilament H

NGF................................. Nerve Growth Factor

NIX................................. BCL2/adenovirus E1B 19 kDa protein-interacting protein 3, BNIP3-like

NMD................................. Neuromuscular Disease

NSAID................................. Nonsteroidal anti-inflammatory drugs

NT-3................................. Neurotrophin 3

NT-4/5................................. Neurotrophin 4/5

NTs................................. Neurotrophic Factors

Opa1................................. OPA1 Mitochondrial Dynamin Like GTPase

P75NTR................................. Neurotrophin Receptor P75

PBS................................. Phosphate Buffered Saline

PCR................................. Polymerase Chain Reaction

PD................................. Parkinson’s Disease

PI3K................................. Phosphoinositide 3-Kinase
PINK1............................. PTEN-induced kinase 1
PKC.............................. Protein Kinase C
PLC-γ............................ Phospholipase C-γ
PO................................. Oral Administration
ROS............................... Reactive Oxygen Species
sALS.............................. sporadic Amyotrophic Lateral Sclerosis
SBMA............................. Spinal-Bulbar Muscular Atrophy
SNPH............................. Syntaphilin coding gene
SOD1............................. Superoxide Dismutase 1
TOM20............................. Translocase of the Outer Membrane 20
TOMM20.......................... Translocase of the Outer Mitochondrial Membrane 20 Antibody
Trk............................... Tropomyosin (Tyrosine) Receptor Kinase
TRPC............................. Transient Receptor Potential Cation Channels
VACHT........................... Vesicular Acetylcholine Transporter
Voxel............................. Volumetric Pixel
CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

The vastly polarized nature and unremitting energy demands of nervous tissue leave neurons susceptible to negative effects of dysfunctional mitochondria. Put simply, the relationship between mitochondria and the neuron is complex, precise, and critical. Neuronal function and survival is dependent on mitochondrial dynamics. In the most basic sense, mitochondria facilitate metabolic control, calcium homeostasis, and the production of ATP. Properly functioning mitochondria mediate synaptic homeostasis by maintaining and generating axonal and synaptic membrane potentials, mobilizing synaptic vesicles for exocytosis, sequestering Ca\(^{2+}\) stores during the post-action potential period, regulating synaptic docking, and promoting neurotransmitter release. (Sheng and Cai, 2012). Further, mitochondria maintain neuron function and survival by energetically supporting the development of the actin cytoskeleton, microtubule motor trafficking, vesicular recycling, and, if necessary, apoptosis. Mitochondria also are self-regulating through internal processes of fission, fusion and mitophagy (Chevalier-Larsen and Holzbaur, 2006; Cozzolino and Carri, 2012; Jiang et al., 2015; Kubli Dieter and Gustafsson, 2012; Misgeld and Schwarz, 2017; Saxton and Hollenbeck, 2012; Sheng and Cai, 2012; Su et al., 2010).

Neuronal survival is dependent on precise maintenance of healthy mitochondrial populations and continual clearance of aged and dysfunctional populations. A lack of this populational homeostasis is detrimental to nonmitotic cells such as neurons. When mitochondria fail to maintain their regulatory functions, the intracellular environment may become excitotoxic thus exposing the cell to unsustainable oxidative stress. Unchecked, these conditions may create a domino-like effect in surrounding mitochondria populations resulting in the failure of metabolic
processes, exposure to reactive oxygen species (ROS), and ultimately apoptotic cell death (Chevalier-Larsen and Holzbaur, 2006; Cozzolino and Carri, 2012; Embacher et al., 2001; Jiang et al., 2015; Kim et al., 2007). Anterograde and retrograde axonal transport of mitochondria, to and from the pre-synaptic terminal, is critical to maintain cell integrity by removing dysfunctional mitochondria and repopulating existing stores (Misgeld and Schwarz, 2017; Sheng and Cai, 2012). The process of axonal transport itself is not possible without properly functioning mitochondria, regardless of the cargo. Compounding the sensitivity of neural health that is dependent on this recycling process, mitochondria are more susceptible to the effects of ROS production due their lack of protective histone proteins to store their internal genome. Additionally, the presence of highly oxidizable proteins in the electron transport chain and the overall architecture of the dual-membrane makes mitochondria vulnerable to ROS exposure, thus requiring mitochondrial life and death cycles to be precisely orchestrated. (Kim et al., 2007).

Thus, the control of mitochondrial fission, fusion and mitophagy dynamics is paramount. Disrupted fission, fusion and mitophagy cycles results in accumulation of damaged mitochondria and exposes the cell to increasing ROS production, sustained loss of ATP production, the inability to buffer Ca\(^{2+}\) in neurons, and results ultimately in the onset of apoptotic cascades.

Four main processes of mitochondrial homeostasis support neuron function: mitochondrial transport, mitochondrial anchoring, fission/fusion dynamics, and mitophagy. Defects in any one of these systems is detrimental to neuron health and survival. Research now suggests that our understanding of the importance of mitochondrial dynamics and the regulation of mitochondrial transport within the neuron have historically been grossly underestimated. Determining the underlying physiology supporting mitochondria production, maintenance, and degradation, as well as how these pathways interact in a disease states, is critical for the development of future
therapeutic treatments in many neurodegenerative diseases. The following is a brief review of mitochondrial dynamics, or mitostasis, in neurons. Consideration of each of these fundamental processes is necessary to understand and deeply appreciate the complex relationship between mitochondrial function and neuron survival.

Mitochondrial Transport

To meet the energy requirements of the neuron, mitochondria function in both a mobile and stationary fashion. Neurons, and in particular motor neurons, are morphologically large and energetically demanding. They are comprised of four major compartments: the soma, dendrites, axon, and synaptic terminal. The distribution of mitochondria, both mobile and stationary, is not treated equally among each compartment. The energy needs of each compartment varies throughout the neuron’s life, and a bi-directional anterograde and retrograde transport system helps to address energy demands by distributing mitochondria where metabolically needed. The pathways regulating mitochondrial mobility are dynamic and complex but facilitate mitochondrial adaptation to energy requirements. The most widely accepted model of mitochondria distribution dynamics in neural systems assumes newly synthesized mitochondria arise in the soma and are anterogradely transported down the axon to the presynaptic terminal. In this model, aged and dysfunctional mitochondria are retrogradely transported back to the soma for lysosomal degradation (Chevalier-Larsen and Holzbaur, 2006; Jiang et al., 2015; Lin et al., 2017; Misgeld and Schwarz, 2017; Saxton and Hollenbeck, 2012; Sheng and Cai, 2012).

Interestingly, minimal research actually exists to support this model. Still, despite the lack of evidence, this model is the most widely accepted (Saxton and Hollenbeck, 2012; Sheng and Cai, 2012). It is reasoned that areas requiring higher ATP demand are more densely populated with mitochondria. In adult neuromuscular systems, these regions would include the neuromuscular junction, synapse, and the Nodes of Ranvier along the axon (Sheng and Cai, 2012).
Bidirectional transport of mitochondria is facilitated by a cytoskeleton framework composed of microtubules, actin, and intermediate filaments. Polymerized αβ-tubulin dimers create a framework of microtubules arranged according to polarity in the anterograde and retrograde directions (Chevalier-Larsen and Holzbaur, 2006). The cytoplasmic motor protein dynein transports cargo towards the minus end of microtubules, and the motor protein kinesin transports cargo towards the plus end of microtubules. Exposed β-tubulin directs microtubules towards the positive end and results in anterograde transport to the axon terminal. Microtubules terminating in α-tubulin direct transport towards the negative end and result in retrograde transport from the presynaptic terminal to the soma (Chevalier-Larsen and Holzbaur, 2006; Sheng and Cai, 2012). Transport of mitochondria along the axon microtubules is considered to be a long-range process requiring ATP hydrolysis and is mediated by motor proteins complexed with adaptor proteins.

Anterograde transport of mitochondria requires mitochondrial coupling to plus-oriented kinesin motor protein complexes. The kinesin motor complex is composed of two heavy chain proteins and two light protein chains. The heavy chains drive the motor complex while the light chains bind to intracellular cargo, which includes mitochondria (Chevalier-Larsen and Holzbaur, 2006; Saxton and Hollenbeck, 2012; Sheng and Cai, 2012). Milton is a well-characterized adaptor protein which links the Rho family GTPase protein Mitochondrial Rho (MIRO) located on the outer mitochondrial membrane to the cargo binding domain of the kinesin motor domain. In Drosophila, mutations of the Milton and Miro genes impairs anterograde transport of mitochondria and depletes synaptic populations (Górska-Andrzejak et al., 2003; Guo et al., 2005; Sheng and Cai, 2012; Stowers et al., 2002; Su et al., 2010). In contrast, the nature of the mechanics driving retrograde transport of mitochondria are unknown. Cytoplasmic dynein is considered the major motor complex responsible for long-range retrograde transport of...
mitochondria. Dynein is composed of two heavy chains which drives motility and several intermediate protein chains, light intermediate protein chains and light protein chains presumed to associate with cargo. Dynactin works as an adaptor protein and allows for the binding of cargo to the motor complex (Chevalier-Larsen and Holzbaur, 2006). Very few adaptor proteins that contribute to retrograde axonal transport of mitochondria have been identified. However, a loss in Miro gene expression has been show to impair retrograde dynein transport of mitochondria, and the dynein-associating adaptor protein dynactin was found to associate with mitochondria in *Drosophila* (Pilling et al., 2006; Russo et al., 2009; Sheng and Cai, 2012). Thus, Miro likely plays a bidirectional role in the transport of mitochondria over long distance, yet the specific mechanisms of this transport are not well understood.

Transport of mitochondria is not solely dependent on microtubules. Actin monomers are thought to facilitate mitochondrial transport over short distances. Actin ultimately forms the cytoskeleton framework and is anchored by filaments on the cell periphery. Actin filaments likely make up the transport network of the terminal, and movement is facilitated by myosin motors (Sheng and Cai, 2012). Although an interaction between mitochondria and myosin has not been demonstrated to date, myosin motors may be responsible for the transition of mitochondria from the microtubules to synapse (Sheng and Cai, 2012). It is important to recognize that regulation of mitochondria transport may be dependent on more than motor protein binding and is likely impacted by intracellular levels of calcium, production of ATP, fission/fusion dynamics, cell signaling from the periphery, and the process of mitophagy. Further, transport and degradation mechanisms under normal physiological conditions may differ greatly within a state of pathology.
Mitochondrial Docking

Stationary mitochondria make up the majority of the mitochondrial population. These mitochondria are anchored to the cytoskeleton framework throughout the dendrites, soma, axon, and axon terminals of neurons. Mitochondria are thought to be recruited to the pre-synapse of neurons in response to intracellular calcium and ATP/ADP levels, while physical docking of mitochondria may be regulated by neurotrophic signaling. However, little is understood regarding how these intrinsic mechanisms coordinate the switch from mitochondrial transport to docking. As the pre-synapse depletes energy stores, intracellular ATP/ADP levels change. Changes in ATP levels regulated by mitochondria drive motor machinery velocity during transport, and an increase in intracellular ADP signals for mitochondrial docking (Sheng and Cai, 2012). Further, mitochondrial recruitment is regulated by calcium levels. Intracellular Ca$^{2+}$ influx inhibits motility, and this process is likely regulated by the outer mitochondrial membrane protein Miro. Miro has two EF hand Ca$^{2+}$ binding domains and has been identified as a calcium sensing regulator of mitochondrial mobility bidirectionally (Cai and Sheng, 2009). Elevated Ca$^{2+}$ levels alter anterograde and retrograde transport, and mutations to the EF hand domains blocks mitochondrial arrest induced by synaptic activity and glutamate release (MacAskill et al., 2009; Wang and Schwarz, 2009). Mitochondrial docking may also be regulated by the expression of Syntaphilin (SNPH) which binds the outer mitochondrial membrane to microtubules (Mandal and Drerup, 2019). Recently, a SNPH mouse model demonstrated SNPH acts as an axonal docking receptor, and homozygous SNPH expression significantly increases mitochondrial mobility and regulates the proportion of stationary mitochondria (Kang et al., 2008). Additionally, SNPH expression may alter mitochondrial docking in response to the cellular environment. SNPH was shown to selectively releases from microtubules in response to mitochondrial stress (Lin et al., 2017). Research aimed at elucidating the mechanisms of synaptic
docking of mitochondria are slowly progressing, but Miro1 and SNPH are two possible adaptor proteins believed to facilitate this process.

Interestingly, mitochondrial docking may be supported by neurotrophic factor signaling. Focal axonal stimulation with nerve growth factor (NGF) both recruits mitochondrial populations to NGF specific regions on the axon and arrests mitochondrial movement out of the region resulting in accumulating mitochondrial populations (Chada and Hollenbeck, 2004). The neurotrophic effect on mobility is thought to be product of the downstream PI3K signaling pathway (Chada and Hollenbeck, 2004). Further, induction of PI3K and PLC-γ pathway by the binding of brain derived neurotrophic factor (BDNF) to its tyrosine receptor kinase (TrkB) receptor increased mitochondrial arrest by elevating intracellular Ca\(^{2+}\) levels (Su et al., 2014). Understanding the transitions between mobile and stationary mitochondria is a new frontier in neurobiology. Even less is understood about the signaling mechanism regulating mitochondrial density in the neuromuscular junction. Despite continued research, most of the mechanics involved in the transition between mobile and anchored mitochondria are unknown and further complicated by interconnected web of signaling cascades simultaneously promoting transport, fission/fusion and mitophagy in the neuron.

*Fission and Fusion Dynamics*

One key mediator promoting neuron health is the unique processes of mitochondrial fission and fusion. Now more than ever, researchers are reconsidering how and where fission and fusion supports the neuron, and this is largely due to the elaborate architecture of the cell itself. In the classic model of mitochondrial turnover, damaged mitochondria are recycled at the soma after retrograde transport from the presynaptic terminal. However, a neuron’s infrastructure is anything but simple, and this model overlooks the possibility for localized control over
mitochondrial populations. Fission and fusion may promote mitochondrial health locally, quickly responding to changes in intracellular calcium levels and facilitating redistribution of mitochondria across the soma, dendrites, and axon. A local system of mitochondrial regulation promotes survival more effectively and efficiently than simply relying on retrograde transport alone. Understanding the potential mechanisms underlying localized mitochondrial turnover is paramount to understanding mitochondrial dynamics, especially in large peripheral neurons.

Fusion is a process in which healthy mitochondria are able to exchange mitochondrial DNA (mtDNA) with damaged mitochondria in response to increasing stress or energy demands. Fusion promotes protein complementation, mtDNA repair, and mitochondrial distribution (Sheng and Cai, 2012; Westermann, 2010). Mitochondrial fusion is mediated by inner and outer mitochondrial membrane proteins. Inner mitochondrial fusion is mediated by optic atrophy 1 (Opa1), and outer membrane fusion is mediated by Mitofusion 1 and 2 (MFN1/MFN2) (Misgeld and Schwarz, 2017; Sheng and Cai, 2012; Westermann, 2010). Fission segregates dysfunctional mitochondria and allows damaged mitochondria to generate a daughter organelle. The process of fission supports the mitochondrial population along the cytoskeleton while promoting mitochondrial clearance, mitophagy and apoptosis (Sheng and Cai, 2012; Westermann, 2010). Mitochondrial membrane fission requires the GTPase dynamin related protein 1 (Drp1) (Misgeld and Schwarz, 2017; Sheng and Cai, 2012; Westermann, 2010). A loss in fission or fusion dynamics and/or mutation to regulating proteins, has resulted in numerous neurological defects and has also demonstrated an interdependent relationship between mitochondrial morphology and transport (Anagnostou and Hepple, 2020; Balog et al., 2016; Chen and Chan, 2009; Sheng and Cai, 2012; Westermann, 2010).
The relationship between fission, fusion and transport is complex, and has yet to be fully characterized. Altered expression in fission- and fusion-mediating proteins demonstrates their role in mitochondrial function and distribution. Defects in fission mechanics due to Drp1 dysfunction leads to altered mitochondrial distribution throughout compartments of cultured hippocampal neurons, and results in altered dendritic synapse and spine populations (Li et al., 2004). Verstreken et al. (2005) aimed to assess mitochondrial dynamics in the neuromuscular junction without altering soma mitochondrial populations. This was achieved by isolating Drp1 mutants in Drosophila. The study found that mitochondria expressing Drp1 mutations are physiologically functional when compared to controls. However, mitochondria failed to localize within the pre-synapse, were unable to mobilize reserve pool vesicles, were deficient at Ca\textsuperscript{2+} buffering, and altered neurotransmission was exhibited (Verstreken et al., 2005). Further, fission- and fusion-deficient neurons may be unable to distribute mitochondria effectively. For example, suppressing expression of the retrograde transport protein dynein affects fission dynamics and mitochondrial morphology (Verstreken et al., 2005). Dynein expression may regulate cytoplasmic Drp1 recruitment, and play a role in the redistribution and morphology of mitochondrial populations undergoing the process of fission (Varadi et al., 2004). In a Charcot-Marie-Tooth neuromuscular disease model, disease pathophysiology of cultured dorsal root ganglion neurons illustrated altered transport of mitochondria both anterogradely and retrogradely as a result of MFN2 mutated protein expression, important for mitochondrial fission (Balogh et al., 2007). The interdependent relationship between mitochondrial morphology and transport was further supported by a study of cultured neurons from MFN2 knockout mice. Misko et al. provides evidence that a MFN2 fusion protein directly interacts with adaptor transport proteins Miro1 and 2, which links mitochondria to microtubules motor complexes.
MFN2 directly interacts with Miro 1 and 2, and loss of MFN2 results in increasing populations of stationary mitochondria and disrupted axonal transport (Misko et al., 2010). Further, these experiments demonstrated that interaction between adaptor proteins and fusion proteins are both present and selective. Coimmunoprecipitation experiments demonstrated MFN2 interactions with adaptor proteins favors Miro2 over Miro1 (Misko et al., 2010). Misko et al. illustrates how interconnected mitochondrial dynamics are, and more than likely, how crosstalk is dependent on several physiological signals and selectively addresses the needs of the neuron through multiple pathways.

*Mitophagy*

The final and most critical way in which mitochondria support the neuron is through the regulatory events which facilitate the elimination of dysfunction mitochondria. Dysfunctional mitochondria may be morphologically altered (fragmented, swollen etc.), membrane potential deficient, ATP deficient, unable to sequester Ca\(^{2+}\), ROS releasing, express mutated mtDNA and other proteins, or simply may be ageing. However, any of these characteristics of dysfunctional mitochondrial are detrimental and impair the organelle’s ability to meet energy demands. Initially, the term mitophagy was coined for this cellular process of mitochondrial self-degradation, but recent advances in scientific research now provide evidence that mitophagy is just one of several mechanism regulating mitochondrial turnover.

Current research suggests dysfunctional mitochondria are degraded through several processes, each specific to the local environment and degree of dysfunction. The mechanics involved in each process of degradation are dependent on the cellular environment, and mitophagy is just one process of elimination. Further complicating our understanding of mitophagy are several large setbacks in research characterizing these mechanisms. The field has come far in
understanding the pathophysiology of this process, but leading hypotheses leave much to be desired in the interpretation of the regulatory mechanisms promoting mitochondrial clearance. Dysfunctional mitochondria often lack a membrane potential, which drastically complicates the process of visualizing mitochondria undergoing the clearance process. Further, leading hypotheses on mitochondrial clearance often are at odds with one another, are poorly translated across in vitro and in vivo studies, and results vary greatly according to the individual research model used.

In general, selective autophagy requires the presence of an autophagy receptor (typically cytosolic). This receptor interacts with proteins linking the autophagy receptor to the microtubules, autophagosome, and targeted cargo (Evans and Holzbaur, 2020; Misgeld and Schwarz, 2017). Mitophagy is triggered by the accumulation of damaged mitochondria. The first and best characterized mitophagy regulators are PTEN-induced putative kinase protein 1 (PINK1) and cytosolic E3 ubiquitin ligase Parkin, of the PINK1-parkin pathway. Depolarization of the mitochondrial membrane results in accumulation of PINK1 on the outer mitochondrial membrane. Increasing accumulation and autophosphorylation of PINK1 then activates parkin and results in the phosphorylation of several substrates downstream, including ubiquitin, Miro and Mfn1 and 2 (Evans and Holzbaur, 2020; Misgeld and Schwarz, 2017; Sheng and Cai, 2012). Activated parkin is recruited to the surface as it binds to phosphorylated ubiquitin and Miro (Misgeld and Schwarz, 2017). Together, the phosphorylation of ubiquitin is driven by the PINK1-parkin pathway and ubiquitination of the outer mitochondrial membrane signals mitochondria for autophagy receptor-mediated degradation (Evans and Holzbaur, 2020; Misgeld and Schwarz, 2017).
In contrast to the PINK1-parkin pathway which recruits autophagy receptors, the autophagy receptor may also be expressed directly on the outer mitochondrial membrane. The Nip-like protein X (NIX) is an autophagy regulator expressed on the outer mitochondrial membrane. NIX phosphorylation is mediated by an unknown kinase, which recruits the autophagosome directly to the outer mitochondrial membrane, independent from the PINK1-parkin pathway (Evans and Holzbaur, 2020). Alternatively, lipid expression on the outer mitochondrial membrane was also shown to mediate mitophagy. An inner mitochondrial membrane phospholipid, Cardiolipin, is externalized to the outer mitochondrial membrane in dysfunctional mitochondria (Evans and Holzbaur, 2020). Accumulation of cardiolipin directly recruits the autophagosome by binding to the microtubule-associated protein, 1A/1B-light chain 3 (LC3) (Evans and Holzbaur, 2020). Further, parkin is not the only E3 ligase of the outer mitochondrial membrane. In a parallel path, mitochondrial ubiquitin ligase 1 (MUL1) recruits the autophagosome by binding Gamma-aminobutyric acid receptor-associated Protein (GABARAP) (Evans and Holzbaur, 2020). Interestingly, MUL1 is regulated by fission mediator DRP1 and fusion mediating proteins Mfn1 and 2 (Evans and Holzbaur, 2020; Sheng and Cai, 2012).

The selective mitophagy pathways summarized above are just a subset of mechanisms facilitating mitochondrial clearance. Mitochondrial turnover along the axon and within the distal axon terminals are likely subject to entirely different models of clearance. The two leading hypotheses are a non-selective pathway of clearance and the formation of mitochondrial derived vesicles (MDV). In non-selective mitochondrial clearance model, elimination of fragmented mitochondria in the axon terminal is dependent on retrograde transport. Mitochondrial fragments are engulfed in autophagic vacuoles and retrogradely transported back to the soma, for degradation by mature lysosomes along the axon and within the soma (Cheng et al., 2015; Evans
and Holzbaur, 2020). Autophagic vacuoles recruit dynein for transport back to the soma, and disruption of dynein transport results in accumulating vacuoles (Cheng et al., 2015). Most recently, a novel mechanism for mitochondrial clearance was demonstrated through the formation of MDVs. Mitochondrial derived vesicles allow for partial elimination of mitochondria, independent of fission and autophagy (Evans and Holzbaur, 2020; Martinez-Vicente, 2017; Sugiura et al., 2014). Budding of the mitochondrial membrane forms MDVs in response to oxidative stress and are targeted to both lysosomes and peroxisomes (Evans and Holzbaur, 2020; Martinez-Vicente, 2017; Sugiura et al., 2014). The formation of MDVs is an important link in the current model of mitochondrial dynamics. MDV studies suggest this process is fast acting under cellular stress and supports the preservation of existing mitochondria instead of the promotion of degradation (Evans and Holzbaur, 2020; Martinez-Vicente, 2017; Sugiura et al., 2014). A local mechanism for mitochondrial turnover, which preserves mitochondria, promotes degradation of organelle contents, and is independent of autophagy, is intriguing. This is particularly true when considering the health and maintenance of mitochondria that are located far away from the soma and anchored to the axon terminals. The events that promote mitochondrial clearance appear to be just as dynamic and diverse as the organelle itself.

**Mitochondrial Dynamics in Pathology**

The cellular cascades and molecular proteins which regulate mitochondrial dynamics demonstrate unparalleled crosstalk in their signaling mechanisms. Further, each signaling cascade promoting mitochondrial distribution and survival seems to be regulated by a multiplicity of factors and adapts according to the environment, transportation, and energy demands of the cell at any given time. In short, mitochondrial dynamics are as diverse as the neurons they support, and sustaining dynamics requires equally diverse signaling mechanisms. Unsurprisingly, dysfunctional mitochondria are heavily cited as underlying neurodegenerative
and neuromuscular diseases (NMD) such as Amyotrophic Lateral Sclerosis, Spastic Paraplegia, Charcot-Marie-Tooth, Alzheimer’s disease, Huntington’s disease and Parkinson’s disease (Borgia et al., 2017; Chen and Chan, 2009; Chevalier-Larsen and Holzbaur, 2006; Correia and Moreira, 2018; Cozzolino and Carrì, 2012; Jiang et al., 2015; Sasaki and Iwata, 2007; Saxton and Hollenbeck, 2012; Stavoe and Holzbaur, 2018).

Mitochondria irregularities are observed in almost all neurodegenerative diseases, but whether mitochondria dysfunction is a detrimental bi-product or causative factor is still largely unknown. For example, Alzheimer’s disease (AD) is characterized by progressive memory deterioration, synaptic connectivity loss and the accumulation of amyloid plaques and neurofibrillary tangles. Recently, research has demonstrated mitochondrial dynamics play a significant role in the pathophysiology behind AD progression. Mitochondrial populations in AD exhibited altered metabolism, reduced cytochrome C production, protein modification, and prominent oxidative stress prior to the appearance of clinical symptoms (Correia and Moreira, 2018). Further, mitochondrial dynamics appear to be altered, and accumulating mitochondria with altered morphology have been reported and are thought to be the byproduct of compromised fission/fusion, mitophagy and axonal transport mechanisms (Chen and Chan, 2009; Correia and Moreira, 2018). In another example, Parkinson’s Disease (PD) is characterized by the progressive loss of dopaminergic neurons. PD studies suggest mitochondria play a significant role in disease pathology. PD studies have demonstrated mitochondrial fragmentation, dysregulated mitochondrial homeostasis, oxidative stress, altered fission/fusion dynamics and a direct link between the PINK1-parkin pathway and familial forms of PD (Chen and Chan, 2009; Correia and Moreira, 2018). Mutations to fusion protein Opa1 results in optic nerve degeneration, while mutations to Mitofusion proteins result in Charcot-Marie-Tooth disease.
(CMT) and the degeneration of peripheral neurons (Chen and Chan, 2009; Misgeld and Schwarz, 2017). Finally, spinal and bulbar muscular atrophy (SBMA) is a disease of the lower motor neurons caused by polyglutamine expansion in androgen receptors. Skeletal muscle biopsy specimens from SBMA patients demonstrated altered mitochondrial morphology and increased mitophagy (Borgia et al., 2017).

Changes in mitochondrial morphology, transport and mitophagy have been extensively studied in disease models for amyotrophic lateral sclerosis (ALS) as well (Boillée et al., 2006; Cozzolino and Carri, 2012; Evans and Holzbaur, 2019; Jiang et al., 2015; Ruffoli et al., 2015; Su et al., 2010). ALS is a fatal neuromuscular disease, characterized by progressive motor nerve degeneration and resulting systemic atrophy of the peripheral nervous system, spinal cord, and brainstem. ALS research models provide striking evidence of the multifaceted strain mitochondrial dysfunction puts on pathophysiology. Further, studies aimed at the development and implementation of therapeutic drugs clearly demonstrates that treatments will likely need to target several mechanisms of dysfunction simultaneously. ALS exist in two forms: sporadic (sALS) and familial (fALS). Familial ALS demonstrate inheritance patterns, and mutations to superoxide dismutase 1 (SOD1) gene locus have contributed greatly to our understanding of fALS (Cozzolino and Carri, 2012; Jiang et al., 2015). Sporadic ALS is the most prevalent form, and the underlying cause of disease onset is unknown. Among the several leading hypothesis supporting ALS pathology, mitochondrial dysfunction is thought to play a role in oxidative stress, cytoskeleton alterations, protein aggregation, dysfunctional mechanism of mitophagy, transport deficits and neuromuscular junction alterations all of which are associated with the disease pathology (Cozzolino and Carri, 2012; Evans and Holzbaur, 2019; Jiang et al., 2015; Ruffoli et al., 2015; Su et al., 2010).
Mitochondrial dysfunction is clearly evident in ALS patient histology, and alterations to mitochondrial morphology throughout motor neurons and skeletal muscle of ALS patients is a pathological hallmark indicating the disease (Cozzolino and Carriè, 2012; Ruffoli et al., 2015; Su et al., 2010). Changes in histological morphology serve as strong indicators for altered function. Biopsies from ALS patients suggest disrupted regulation of mitochondrial Ca\(^{2+}\) within the nerve terminal, and various alterations to respiratory chain complexes are found in post-mortem brain, spinal cord and skeletal muscle tissues (Cozzolino and Carriè, 2012; Jiang et al., 2015; Su et al., 2010). The development of mutant SOD1 mouse models have greatly contributed to our understanding of mitochondrial dysfunction in ALS and allowed for the specific development of studies targeting mitochondrial dynamics. Mutant SOD1 models, both in vivo and in vitro, have helped to elucidate the dynamic role of mitochondria in oxidative stress, calcium regulation, motility, and cell death in ALS patients (Cozzolino and Carriè, 2012; Jiang et al., 2015; Ruffoli et al., 2015; Su et al., 2010).

Research aimed at understanding mitochondrial dysfunction in disease pathology is of utmost importance. Not only does this research aim to develop therapeutic treatments, but it demonstrates the complexity of mitochondrial function. Put simply, properly functioning mitochondria and dysfunctional mitochondria require much of the same machinery but respond differently to physiological conditions within the cell. The mechanism involved in mitochondrial transport are only beginning to be understood, and to date over 20 molecular adaptors have been discovered to facilitate this process. Each is regulated by various and specific physiological signaling processes, working in conjunction and sometimes in opposition, while responding to changes in the cellular environment. Mitochondrial dynamics are dependent on cell age, cell architecture and cell health, while also facilitating each of these processes, further complicating
our understanding. One thing is clear, if mitochondria do not function properly, neither does the neuron. Changes to cell signaling cascades that alter intracellular calcium levels may ultimately have an effect on mitochondria transport, anchoring and mitophagy. Deficits in axonal transport, will likely have a resulting effect on mitochondrial localization and transport dynamics. Disruption to mechanism of autophagy and programmed cell death will likely have an effect on mitochondrial populations and health. Further, mitochondrial dysfunction is prominent in disease states, but the degree of dysfunction is as dynamic as the pathways facilitating it. In short, mitochondrial dysfunction may be a byproduct of disease states or a causative factor. Research has a long way to go in distinguishing between the two possibilities. Our understanding of mitochondrial dynamics has come far in the past twenty years, but a greater effort to characterize the cellular signaling cascades that promote mitochondria is necessary moving forward. Mitochondria serve an essential role in the neuron, and researchers are now considering whether mitochondrial dynamics are regulated by equally an essential mechanism: neurotrophic signaling.

**Neurotrophic Signaling**

Neurotrophins (NTs) play a significant role in neurogenesis, transport, and synaptic plasticity through the activation of receptors and secondary signaling cascades. The essential roles of mitochondria in the neuron have led scientists to consider whether neurotrophins play a regulating role in mitochondrial dynamics. NTs are functionally complex subset of trophic factors, and expression of NTs support the neuronal cell via axonal and dendritic growth, development of synaptic patterning, neurotransmitter release, synaptic plasticity, and mitochondrial regulation (Bothwell, 2016; Chao, 2003; Delezie et al., 2019; Su et al., 2010). Four NTs have been identified in mammals and include NGF, BDNF, Neurotrophin-3 (NT-3) and Neurotrophin-4/5 (NT-4/5). NTs function in both neural and nonneuronal tissue and are
active during both embryonic development and throughout adult physiology (Bothwell, 2016). NT signaling requires the activation of two classes of transmembrane-receptors: the tyrosine kinase receptors (Trk) and a 75 kDa pan-neurotrophic receptor (P75NTR).

Neurotrophins can be secreted in both pro-neurotrophin and mature form. All NTs express a pro-neurotrophin form and subsequently are cleaved to reveal an N-terminal pro-domain and C-terminal. The pro-neurotrophin form can be cleaved during the secretory pathway or post-secretion (Bothwell, 2016; Chao, 2003; Huang and Reichardt, 2003). The cell surface tyrosine kinase receptors include the receptors TrkA, TrkB, and TrkC. The relationship between neurotrophin and receptor is complex and highly specific. For example, the P75NTR receptor is activated by all four neurotrophins, while Trk receptors are far more selective and only bind to the mature neurotrophin form. Among the Trk receptors, NGF binds to TrkA, NT-3 binds to TrkC, and BDNF and NT-4/5 binds to TrkB (Huang and Reichardt, 2003). NT activation of their individual receptors have been described as a “ying-yang” signaling, as these receptors are often co-expressed, but individual neurotrophic binding often activates opposing pathways (Bothwell, 2016; Lu et al., 2005). For example, activation of receptor TrkA stimulates axonal growth and cell survival, while activation of P75NTR facilitates axonal degeneration and cell death (Bothwell, 2016; Singh et al., 2008). In another example, hippocampal expression of BDNF activates P75NTR receptors and promotes long term depression, while BDNF-TrkB activation promotes long term potentiation (Bothwell, 2016; Lu et al., 2005).

Neurotrophins are released by the post-synaptic target tissue and require both endocytosis and retrograde transport to mediate signaling cascades within the soma. The mechanism promoting the release and transport of NT factors have been a subject of research for decades. Several models supporting retrograde transport of NT have aimed to elucidate the mechanism behind this
signaling processes (Ascano et al., 2012; Ginty and Segal, 2002; Harrington and Ginty, 2013; Scott-Solomon and Kuruvilla, 2018). In the ‘domino’ model of retrograde signaling, NTs will bind to their respective receptors, independently of vesicular transport, and induce a wave of receptor phosphorylation back to the soma (Ginty and Segal, 2002). In the ‘retrograde effector’ model, Trk binding initiates several signaling pathways locally in the distal neuron and initiate transport of the NT/Trk complexes back to the soma (Ginty and Segal, 2002).

Currently, the leading hypothesis supporting neurotrophic release and regulation is the ‘signaling endosome’ model (Ascano et al., 2012; Cosker and Segal, 2014; Ginty and Segal, 2002; Harrington and Ginty, 2013; Scott-Solomon and Kuruvilla, 2018). In the signaling endosome model, NT/Trk binding in the distal neuron promotes signaling cascades both retrogradely and locally, undergoes microtubule-dependent transport, promotes transcription within the soma, and requires clathrin-mediated endocytosis or Pincher-mediated micropinocytosis (Cosker and Segal, 2014; Ginty and Segal, 2002; Scott-Solomon and Kuruvilla, 2018; Yamashita and Kuruvilla, 2016). The activated NT/Trk signaling endosome further controls the nuclear transactivation of genes in the soma (Bothwell, 2016; Chao, 2003; Cosker and Segal, 2014; Ginty and Segal, 2002). The release of NT’s by target tissues facilitate dimerization of Trk receptors which stimulate a complexity of intracellular cascades. To date, the formation of NT/Trk signaling endosomes are shown to regulate neuronal growth, synaptic plasticity, apoptotic signaling, synaptic formation, dendritic formation, and neuronal competition in embryonic development (Ascano et al., 2012; Harrington and Ginty, 2013; Scott-Solomon and Kuruvilla, 2018).

Neurotrophic signaling is known to promote development, but their effects are seen well into adulthood as well. In mature neurons, neurotrophin signaling maintains neuron phenotypes, cellular transport, neurotransmission and regulate plasticity (Bothwell, 2016; Chao, 2003; Yoshii
and Constantine-Paton, 2010). Likely, the effects of NT signaling on intracellular cascades is subject to changes in the levels of NT expression or NT gene mutations. In fact, haploinsufficiency of several neurotrophins is well characterized in research through the use of developed mouse models. Heterozygous expression of neurotrophins NGF, BDNF and NT3 have all demonstrated neuron loss in the peripheral nervous system, and both NT3 and BDNF haploinsufficient mice have a reduction or loss in mechanoreceptor sensitivity (Bianchi et al., 1996; Chao, 2003; Crowley et al., 1994; Ernfors et al., 1994a, 1994b). More recently, neurotrophic support has been being characterized in disease models. For example, NT signaling is altered by several psychiatric treatments and their role in intracellular signaling must be considered further in the developments of therapeutics treatments (Chao, 2003). A single amino acid change in the pro-domain of BDNF, from valine to methionine has been linked with depression, bipolar disorder and schizophrenia (Chao, 2003; Neves-Pereira et al., 2002; Sen et al., 2003; Sklar et al., 2002). Additionally, mutations in BDNF and P75NTR receptors have been characterized in neurodegenerative diseases such as AD (Chao, 2003; Hu et al., 2002; Ventriglia et al., 2002). A disruption in retrograde transport has been demonstrated in AD models, and a reduction in both NGF and TrkA receptors levels were found within the basal forebrain (Counts et al., 2004; Salehi et al., 2006). Further, deficits in endosomal retrograde trafficking were demonstrated in both ALS and CMT disease models (BasuRay et al., 2010; Devon et al., 2006). The roles of neurotrophic signaling in disease pathology are still undefined, but by far the most well-characterized neurotrophin is BDNF and its full length, high-affinity receptor TrkB. BDNF is most often secreted as proBDNF and proteolytically cleaved extracellularly before binding to the receptor TrkB (Mowla et al., 2001). The homodimerization of TrkB receptors results in cross tyrosine phosphorylation of intracellular domains, and phosphorylation of TrkB
receptors stimulates multiple signaling cascades, including phospholipase-C-γ (PLC-γ), phosphatidylinositol-3 kinase (PI3K) and mitogen-activated protein kinase (MAPK) (Bathina and Das, 2015; Cosker and Segal, 2014, 2014; Su et al., 2014; Yamashita and Kuruvilla, 2016). Through a signaling endosome, BDNF/TrkB-mediated activation of cell signaling cascades promote several neuroprotective effects locally and downstream. Phosphorylation of TrkB promotes the PLC-γ pathway, which in turn generates inositol triphosphate (IP3) and diacylglycerol (DAG). IP3 increases intracellular Ca^{2+} concentrations, while DAG activates protein kinase C (PKC). The PLC-γ pathway results in the activation of calcium-sensing enzymes, transcription factors and ion channels, while also promoting synaptic plasticity and neuronal growth (Bathina and Das, 2015; Cosker and Segal, 2014; Huang and Reichardt, 2003; Meng et al., 2019; Yoshii and Constantine-Paton, 2010). Activation of the PI3K pathway is antiapoptotic, increases protein translation, and promotes cell survival (Bathina and Das, 2015; Meng et al., 2019). PI3K activates the protein kinase Akt, which phosphorylates downstream targets promoting cell survival and work antiapoptotically to inhibit BCL2 associated death protein (BAD) (Cosker and Segal, 2014; Huang and Reichardt, 2003). PI3K pathways also facilitate the retrograde transport of signaling endosomes which further promote synaptic plasticity and protein translation (Cosker and Segal, 2014; Huang and Reichardt, 2003; Meng et al., 2019). Further, BDNF/TrkB binding mediates cell survival and works antiapoptotically through the MAPK signaling cascade. Ras-mediated induction of MAPK signaling cascades stimulates antiapoptotic proteins expression and activates cAMP response element-binding protein (CREB). MAPK phosphorylation of CREB influences gene transcription, differentiation, and cell survival (Harrington and Ginty, 2013; Huang and Reichardt, 2003; Yoshii and Constantine-Paton, 2010). Overall, BDNF/TrkB signaling promotes neuron survival through
activation of secondary messengers (Figure 1). The neuroprotective effect of BDNF-TrkB signaling on the neuron, has led researchers to now question whether BDNF-TrkB signaling has a role in the neuroprotective dynamics of mitochondria.

Figure 1. A schematic of BDNF-TrkB and BDNF-p75NTR receptor activated pathways. Activation of BDNF-TrkB signaling results in the activation of secondary messenger including the PLCγ, MEK and PI3-K signaling pathways. BDNF-TrkB receptor binding promotes neuron survival, plasticity, and growth. Activation of the p75NTR receptor promotes survival, axon degeneration and cellular apoptosis (Brandt, 2018).
Neurotrophins and Mitochondria

A collection of studies has aimed at demonstrating a possible link between mitochondrial function and neurotrophin signaling. Various isoforms of the TrkB receptor colocalized to the outer mitochondrial membrane in human skeletal muscle, and accumulation of the neurotrophin NT-4 was found in the surrounding mitochondrial populations (Wiedemann et al., 2006). BDNF signaling was shown to modulate oxidative phosphorylation through the activation of the TrkB receptor (Markham et al., 2004). Markham et al. hypothesized a possible link between neurotrophic signaling and neuron metabolism, and these studies demonstrated that BDNF increased ATP synthesis in mitochondria selective to complex I in rat brain tissue samples (Markham et al., 2004). Further, neurotrophic signaling appears to both modulate mitochondrial transport and synaptic docking in neurons. Chada and Hollenbeck proposed NGF-TrkA signaling regulated mitochondrial transport and docking through the activation of the PI3K signaling pathway in sensory axons. To examine this relationship, cultured sensory axons were focally stimulated with NGF covalently conjugated to polystyrene beads (Chada and Hollenbeck, 2004). NGF focal stimulation resulted in local accumulation of mitochondria and had a regulatory effect specific to mitochondrial transport only (Chada and Hollenbeck, 2004). Additionally, inhibition of the PI3K pathway eliminated local regulation of mitochondrial transport by NGF in sensory axons (Chada and Hollenbeck, 2004).

Su et al. aimed to investigate whether BDNF signaling could regulate mitochondrial motility in primary hippocampal neurons. BDNF treatment of cultured hippocampal neurons reduced mitochondrial bidirectional movement along axons and increased stationary mitochondrial populations (Su et al., 2014). BDNF-induced regulation of mitochondrial movement was due to an increased intracellular Ca^{2+} levels (Su et al., 2014). Increasing intracellular levels of Ca^{2+} was a product of the PI3K and PLC-γ signaling pathway and transient receptor potential canonical
(TRPC) channels activity, and was found to be required for BDNF-induced mitochondrial arrest (Su et al., 2014). Further, Su et al. demonstrated BDNF-induced mitochondrial docking was mediated by the expression of the calcium sensing regulator Miro1 on the outer mitochondrial membrane (Su et al., 2014). Miro1 recruits mitochondria to pre-synaptic sites, which then enhanced neurotransmitter release and synaptic transmission (Su et al., 2014). This study was the first to show BDNF/TrkB signaling as a regulator of mitochondrial mobility and demonstrated the resulting enhancement of synaptic transmission.

Contrary to its name, BDNF is synthesized among various tissues types, and more recently the protective roles of BDNF in mitochondrial dynamics have been examined outside of the nervous system. In fact, BDNF/TrkB signaling has proven to be so effective in protecting cells from apoptotic events it has a negative effect on therapeutic treatments for neuroblastoma cells. Neuroblastoma tumors expressing high levels of BDNF are insensitive to chemotherapy treatments (Jaboin et al., 2002). Importantly, neuroblastoma apoptosis is rescued by BDNF/TrkB signaling, and the subsequent activation of the PI3K pathway inhibits chemotherapeutic treatments (Jaboin et al., 2002). Apoptosis was only restored by inhibition of TrkB and PI3K pathways in neuroblastoma tumors (Jaboin et al., 2002). In a recent study of brain microvascular endothelial cells (BMECs), BDNF-TrkB signaling was shown to both support mitochondrial function and induce a mitophagy pathway (Jin et al., 2019). To examine the protective role of BDNF in endothelial cells, Jin et al. induced hypoglycemia, which increases ROS production and prompted apoptosis, in cultured BMECs in order to elucidate BDNF-TrkB signaling mechanism under conditions of oxidative stress.

In other studies, BDNF/TrkB signaling rescued cells from oxidative stress by activating the hypoxia-inducible factor-1α (HIF-1α) and BCL2/adenovirus E1B 19 kDa protein-interacting
protein 3 (BNIP3) mitophagy pathway (Jin et al., 2019). Hyperglycemia resulted in increased mitochondrial permeability transition pore opening, decreased mitochondrial membrane potential, and increased mitochondrial calcium levels (Jin et al., 2019). BDNF treatment prevented morphological changes to mitochondrial structure, which rescued mitochondrial membrane potentials, normalized calcium levels and increased ATP production (Jin et al., 2019). BDNF treatment significantly upregulated the expression of mitochondrial transcription factors and demonstrated a protective role for BDNF signaling in mitochondrial biogenesis (Jin et al., 2019). Oxidative stress was alleviated in BMECs following BDNF treatment through BDNF/TrkB activated mitophagy (Jin et al., 2019). BDNF-TrkB was able to induce mitophagy through TrkB activation of the HIF-1α/BNIP3 complex on the outer mitochondrial membrane (Jin et al., 2019). Together these findings support the hypothesis that BDNF signaling may protect cellular function by promoting mitochondrial clearance through its downstream targets.

*Muscle-Synthesized BDNF*

Neuronal synthesis of BDNF and the subsequent activation of TrkB have been thoroughly characterized throughout the nervous system, but the roles of BDNF synthesized by skeletal muscle are much less understood. Since 2008, the Ottem laboratory has investigated the role of muscle-synthesized BDNF (msBDNF) in motor neuron health, neuromuscular junction morphology and axonal transport using a msBDNF knockout mouse model. These animals display adult-onset phenotypic behaviors similarly found in other neuromuscular disease models. BDNF transgenic mice are experimentally bred to evaluate the role of muscle-synthesized BDNF (msBDNF) in motor neuron health using the Cre-Lox recombinase gene technology. The muscle-synthesized BDNF coding region is removed by the human skeletal actin (HSA) promotor of Cre-recombinase in transgenic mice. Experimentals animals are generated by crossing msBDNF-flox mice with HSA-Cre recombinase positive mice, resulting in offspring
expressing one or both copies of the msBDNF gene. Subsequently, experimental colonies of heterozygous knockout (msBDNF<sup>+/−</sup>), homozygous knockout (msBDNF<sup>−/−</sup>), and control (msBDNF<sup>+/+</sup>) mice are generated.

Analysis of the gastrocnemius (gastroc) muscle in both homozygous and heterozygous knockout showed significant pathology in both muscle and neural tissue types. Histological analysis of the gastroc demonstrated centralized nuclei, fiber splitting, age-dependent fiber loss, and the presence of both hypertrophic and heterotrophic type fibers in skeletal muscle (Figure 2; Taisto et al., 2013). Skeletal muscle myopathy in the gastroc of experimental animals was significantly increased when compared to controls at 30d, 90, 120, 210d (Figure 3; (Taisto et al., 2013).

![Figure 2. Histological analysis of myopathy in gastroc fibers of muscle-synthesized BDNF deficient 120d mice using hematoxylin & eosin (H&E) staining. (A) Myopathy does not appear to be demonstrated in msBDNF control animals at 120d and is considered healthy due to the presence of peripherally located nuclei and a general lack of fiber splitting. (B) Myopathy of the gastroc fibers is demonstrated in heterozygous knockouts and is characterized by the presence of centralized nuclei and split fibers. (C) Myopathy appears the most severe in the gastroc fibers of homozygous knockouts and is characterized by the presence of centralized nuclei, split fibers, and extensive muscle hypertrophy and hypotrophy.](image-url)
Figure 3. Mean percentage of gastrocnemius muscle fibers exhibiting myopathy in control and muscle-synthesized BDNF deficient mice at 30d, 90d, 120d, 180d, and 210d. A significant increase in myopathy is demonstrated in both heterozygous and homozygous knockouts compared to controls across all age groups.

Experimental animals demonstrate significant changes in neuromuscular junction morphology (Figure 4). Immunolabeling of presynaptic proteins in msBDNF−/− mice indicates a significantly smaller mean surface area when compared to controls, and both heterozygous and homozygous knockouts have significantly higher probability for pre-synaptic fragmentation than control animals (Figure 5 Figure 6; (Taisto et al., 2013).
Figure 4. Representative photomicrograph of the neuromuscular junction fragmentation in muscle-synthesized BDNF deficient mice compared to controls at 120d. The neuromuscular junction is delineated by pre-synaptic VAChT (cyan) and post-synaptic AChR (red) immunolabeling. (A) Control animals show intact neuromuscular junction labeling with complete overlap of pre- and post-synaptic surfaces. (B) Heterozygous knockout demonstrates pre-synaptic fragmentation (cyan) characterized by a loss in overlap between surfaces. (C) Homozygous knockouts demonstrate a significant increase in pre-synaptic fragmentation (cyan) and increasing loss of overlap between pre- and post-synaptic surface labeling.

Figure 5. Mean surface area immunofluorescence of pre-synaptic VACHt and post-synaptic AChR immunolabels in the gastroc-associated neuromuscular junction of 120d mice. (A) There is a significant decrease in pre-synaptic VACHt surface area immunofluorescence in homozygous knockouts when compared to controls. (B) There is no significant change to post-synaptic AChR surface area immunofluorescence across all genotypes.
Figure 6. A multiple sample predictor model which measures the mean probability for gastroc-associated neuromuscular junction fragmentation across all genotypes. Mean probability of gastroc-associated neuromuscular junction fragmentation is significantly higher in heterozygous and homozygous knockout animals when compared to controls.

Because our histological findings were characteristic of NMD’s, we next aimed to assess the gastroc-associated motor neurons. Heterozygous and homozygous gastroc-associated neurons demonstrated significantly decreased soma size and dendritic length in 30d and 120d animals (Figure 7; Pomeroy, 2013). These findings suggest that msBDNF may provide neurotrophic support to the neuromuscular junction and promote synaptic strength. We next assessed whether a loss of msBDNF disrupted transport mechanisms along the axon. Hyperphosphorylated neurofilament-H (NF-H-P) is associated with retrograde dynein motors. Distal gastroc-associated neurons show a significant increase in accumulation of NF-H-P in 120d heterozygous and homozygous animals (Figure 9; Dangremond, 2016). Further, using sciatic nerve ligation, Dangremond (2016) demonstrated a significant decrease in NF-H-P accumulation distal to the point of ligation in homozygous knockouts at 120d (Figure 10).
Together, these findings suggest that loss of msBDNF may result in deficits to retrograde transport complexes and that msBDNF is important in the maintenance of the pre-synapse at 120d.

Figure 7. The mean cell soma surface area of the gastroc-associated motorneuron in 30d and 120d animals. (A) Heterozygous and homozygous knockout animals demonstrate a significant decrease in mean soma area when compared to controls at 30d. (B) Heterozygous and homozygous knockout animals demonstrate a significant decrease in mean soma area when compared to controls at 120d.
Figure 8. Mean dendritic length was measured in fluorogold-labeled gastroc-associated lumbar motorneurons at 30d and 120d. A significant decrease in mean dendritic length was found in the lumbar motorneurons of heterozygous and homozygous knockout animals when compared to controls at both 30d and 120d.

Figure 9. Mean surface area NF-H-P immunolabeling in the distal gastroc-associated motorneuron axon at 30d and 120d in muscle-synthesized BDNF deficient mice. (A) There was no significant change in the mean surface area of NF-H-P immunolabeling at the distal axon across all genotypes in 30d mice. (B) There was a significant increase in NF-H-P mean surface area immunolabeling in the distal axon of heterozygous and homozygous animals when compared to controls at 120d.
Figure 10. Mean surface area accumulation of NF-H-P immunofluorescence immediately distal to the point of ligation in 30d and 120d muscle-synthesized BDNF deficient mice. (A) There is no significant difference in NF-H-P mean surface area across all genotypes at 30d. (B) There is a significant decrease in NF-H-P mean surface area accumulation immediately distal to the point of ligation in homozygous knockout mice when compared to controls at 120d.

Dangremond (2016) hypothesized that reduced msBDNF would result in disrupted axonal transport and would subsequently increase mitochondrial density at the pre-synapse. Further findings of this study found significantly decreased labeling of mitochondria in msBDNF−/− knockouts at the axon terminal and a declining trend in msBDNF+/− knockouts (Figure 11). Additionally, an overall decrease in density of mitochondria was found in the post-synapse of msBDNF−/− animals when compared to controls (Figure 11; Dangremond, 2016). Dangremond 2016 proposed that decreased msBDNF may result in reduced mitochondrial trafficking to the axon terminal. However, the study had limitations and the MitoTracker probe used to label density is only sequestered by living mitochondria. Therefore, potentially dysfunctional mitochondria may be accumulating at the axonal terminal and unable to sequester the MitoTracker dye.
Figure 11. Mitochondrial density in the pre- and post-synaptic terminal was measured in gastroc-associated neuromuscular junction of muscle-synthesized BDNF deficient mice. Mean voxels of MitoTracker colocalization with pre-synaptic VACHT and post-synaptic α-bungarotoxin surfaces was measured at 120d. (A) There was a significant decrease in mean MitoTracker-VACHT voxel colocalization at the pre-synaptic surface in homozygous knockouts at 120d. (B) There was no significant difference in mean MitoTracker-α-bungarotoxin voxel colocalization at the post-synaptic surface across all genotypes.

In current studies, we aim to assess the total mitochondrial population and the role of msBDNF in mitochondrial maintenance within the gastroc-associated neuromuscular junction and along the motor neuron axon. To address the potential shortcomings of MitoTracker dye at the axon terminal, mitochondria were co-labeled using both a membrane potential independent dye and immunohistochemistry of the translocase of the outer membrane, TOM20. The combination of a mitochondrial structural protein label and membrane potential independent dye may allow for continued investigation of msBDNF role in mitochondrial population and elucidate mitochondrial transport within the axon.
CHAPTER TWO: GENERATION OF EXPERIMENTAL ANIMALS

Introduction:
To investigate the roles of muscle-synthesized BDNF expression in the maintenance of mitochondrial populations we used a transgenic mouse model. Experimental transgenic animals were generated using the Cre-Lox gene technology from two existing founding colonies at Northern Michigan University: msBDNF-flox mice and HSA-Cre recombinase positive mice. HSA-Cre positive mice express the transgene Cre-recombinase and subsequent promotor human skeletal actin (HSA). BDNF-floxed mice express a 34-basepair Lox-p site flanking the coding region of the BDNF gene. The muscle-synthesized BDNF coding region is removed by the HSA promotor in Cre-recombinase positive mice and is specific to skeletal muscle tissue only.

Experimental animals were generated by crossing msBDNF-flox mice with HSA-Cre recombinase positive mice, resulting in offspring expressing one or both copies of the msBDNF gene. Control animals were negative for HSA-Cre gene and express both copies of the msBDNF coding region flanked by lox-p sites. Generations of experimental breeding resulted in heterozygous knockout (MuscleBDNF+/−), homozygous knockout (MuscleBDNF−/−), and control (MuscleBDNF+/+) mice. The breeding scheme used to generate experimental animals is explained in the following section and further outlined by the breeding schematics provided below (Figure 12).
Figure 12. Cre-Lox gene technology is used to generate muscle-synthesized BDNF deficient mice by eliminating BDNF gene expression in skeletal muscle tissue only. A transgenic mouse expressing the Cre recombinase gene driven by the human skeletal actin (HAS) promoter is crossed with a transgenic mouse expressing the BDNF gene flanked by loxP sites. Experimental animals inherit the Cre recombinase gene and subsequently synthesized the Cre recombinase enzyme in skeletal muscle tissue. The expression of Cre recombinase in skeletal muscle tissue than excises the BDNF sequence flanked by loxP sites.
Figure 13. Transgenic experimental animals were generated through a series of backcrosses to knockout skeletal muscle-synthesized BDNF in male mice. The F1 cross combined an animal expressing the floxed BDNF gene and an animal positive for the Cre recombinase gene. All F1 generated offspring were heterozygous for the floxed BDNF gene, while only a subset of offspring expressed the Cre recombinase gene. Heterozygous floxed BDNF males, who were also positive for the Cre recombinase, were then used as experimental heterozygous knockout (HE KO) animals. Females expressing the same genotype were used as F2 breeders. In the F2 cross, female heterozygous knockout animals were crossed with males expressing the floxed BDNF gene. The F2 offspring generated homozygous knockout (HO KO), heterozygous knockout (HE KO), and control (CO) animals. In the F3 cross, female homozygous knockout animals were crossed with males expressing the floxed BDNF gene. The F3 offspring yielded additional male homozygous knockout animals.
Methods:

Animals were maintained in compliance with the *Institutional Animal Care and Use Committee* approved protocol #311 and *NIH Guidelines for the Care and Use of Laboratory Animals*. Animal husbandry and housing conditions were maintained 365 days a year. To reduce stress, all animals were housed in optimal environmental conditions controlled as follows: temperature (72 °F +/- 4 °C), humidity (55% +/- 15%) and lighting (14-h light/ 10-h dark cycles). Animals were fed ad lib; with water supply always accessible. To promote nesting behaviors and the reduction of stress, environmental enrichment was replenished weekly.

*Generation of Heterozygous Knockout Mice*

PCR analysis was used to identify BDNF-floxed (BDNF\textsuperscript{lox+/-}, Cre\textsuperscript{-/-}) males containing the lox-p coding region and HSA-Cre positive (BDNF\textsuperscript{lox+/-}, Cre\textsuperscript{+/-}) females expressing the Cre-recombinase gene. All first-generation offspring resulted in the heterozygous expression of the BDNF-floxed (BDNF\textsuperscript{lox+/-}) gene. Resulting offspring were heterozygous in expression of Cre-recombinase (BDNF\textsuperscript{lox+/-}, Cre\textsuperscript{+/-}) gene or showed no expression (BDNF\textsuperscript{lox+/-}, Cre\textsuperscript{-/-}) gene. PCR analysis was used to identify all heterozygous knockouts (BDNF\textsuperscript{lox+/-}, Cre\textsuperscript{+/-}). Males heterozygous for BDNF\textsuperscript{lox+/-}, Cre\textsuperscript{+/-} genotype were used as heterozygous knockouts for experimental procedures.

*Generation of Homozygous Knockout Mice*

Females expressing the heterozygous BDNF\textsuperscript{lox+/-}, Cre\textsuperscript{+/-} genotype were used for second generation breeding. Female heterozygous knockouts (BDNF\textsuperscript{lox+/-}, Cre\textsuperscript{+/-}) were crossed with BDNF-floxed (BDNF\textsuperscript{lox+/-}, Cre\textsuperscript{+/-}) males. From the second-generation offspring, males expressing the homozygous BDNF-floxed (BDNF\textsuperscript{lox+/-}, Cre\textsuperscript{+/-}) genotype were used as homozygous knockouts for experimental procedures.
**Generation of Control Mice**

Females expressing the homozygous BDNF\textsuperscript{lox+/+}, Cre\textsuperscript{+/−} genotype were used for third generation breeding. Female homozygous knockouts (BDNF\textsuperscript{lox+/+}, Cre\textsuperscript{+/−}) were crossed with BDNF-floxed (BDNF\textsuperscript{lox+/+}, Cre\textsuperscript{−/−}) males. Resulting male offspring expressing the wildtype BDNF-floxed (BDNF\textsuperscript{lox+/+}, Cre\textsuperscript{−/−}) genotype were used as experimental controls.

**Experimental Genotyping**

PCR analysis was used to confirm the successful implementation of the Cre-lox gene technology in skeletal muscle tissue. Using gel electrophoresis, control animals expressing the wildtype BDNF\textsuperscript{+/+} gene expressed a 2.45 kilobase-pairs (kbp) amplification product in skeletal muscle, kidney, heart, and brain tissue. The activity of tissue specific HSA-Cre recombinase is verified by the amplification of a truncated 950 bp product in skeletal muscle only and unaltered amplification in kidney, heart, and brain tissues (Figure 14). Expression of the HSA-Cre recombinase in Muscle\textsuperscript{BDNF−/−} tissue results in the removal of 1.5 kbp from the BDNF coding exon. The removal of 1.5 kbp from the BDNF coding region is dependent on the presence of the two loxP sites. Floxed alleles express a PCR product 68 base-pairs longer than wildtype BDNF\textsuperscript{+/+} PCR product. Two separate PCR reactions are required to detect the presence or absence of both Floxed BDNF alleles and the HSA-Cre recombinase.

The first PCR reaction distinguishes floxed and non-floxed BDNF alleles based on size using two primers. One primer is specific to the first loxP site and binds upstream. The second primer is specific to the BDNF coding region and binds centrally. Mice expressing two floxed BDNF alleles have an additional 68 base-pairs present in the BDNF coding region and are indicated by one band. Mice expressing one floxed BDNF allele have a shorter amplified product and are indicated by the presence of two bands. Therefore, the amplification of the BDNF PCR reaction distinguishes the floxed allele (487 bp) product from the non-floxed allele (437 bp) product.
second PCR reaction detects the presence or absence of the HSA-Cre recombinase. Non-native to mice, the Cre gene required a set of primers which recognize the internal positive control. The second set of primers was used to detect the presence or absence of the Cre gene. Amplification of the Cre PCR reaction distinguishes the presence of 300 bp housekeeping gene across all animals and the absence or presence of a shorter 100 bp Cre product. Photographs of PCR assay on agarose gel depict the successful amplification of both Cre and BDNF PCR reactions (Error! Reference source not found. Figure 16).
Figure 14. Photograph of amplified BDNF gene PCR assay on agarose gel. (A) Control animals demonstrate the full-length amplification of the BDNF gene in skeletal muscle (2050 bp), kidney, heart, and brain tissues. (B) *Homozygous knockout animals demonstrate a shortened BDNF gene amplification product in only skeletal muscles (975 bp).
Figure 15. Representative photomicrograph of the CRE recombinase gene PCR assay on an agarose gel. The top band represents the internal housekeeping gene that all animals should express (300 bp). The bottom band represents the CRE recombinase gene (100 bp).

Figure 16. Representative photomicrograph of the BDNF gene PCR assay on an agarose gel. Lane 1 depicts BDNF gene expression in a true wildtype, both negative for floxed markers and the CRE recombinase (419 bp). The top band depicted in lane 2 represents the BDNF floxed gene (487 bp). The bottom band depicted in lane 3 represents the BDNF gene that is not floxed (437 bp).
CHAPTER THREE: ASSESSING THE ROLE OF SKELETAL MUSCLE-SYNTHESIZED BDNF IN THE MAINTENANCE OF MITOCHONDRIAL POPULATIONS AT THE NEUROMUSCULAR JUNCTION

Introduction:
As discussed in Chapter 1, the connection between mitochondrial function and pathology may be result of several changes to mitochondrial dynamics. Disruption to transport, impaired clearance machinery, or lack of synaptic docking would all diminish the ability of mitochondria to support the synapse. Mitochondrial populations within the neuron are thought to be most dense in areas with greater energy costs, such as the axon terminal. We reasoned a lack of msBDNF and TrkB receptor activation at the synapse would disrupt signaling pathways and result in mitochondrial dysfunction at the neuromuscular junction. In 2016, Dangremond assessed the role of msBDNF in the maintenance of mitochondrial density at the neuromuscular junction and hypothesized that there would be a significant change in mitochondrial density at the neuromuscular junction in animals deficient in msBDNF. Dangremond (2016) hypothesized a change in mitochondrial population would be due to a combination of axonal trafficking and BDNF-TrkB signaling deficits at the synapse.

Dangremond (2016) found a significant decrease in mitochondrial density at the pre-synapse of motor neurons in msBDNF−/− mice at 120d and a decreasing trend in mitochondrial populations in the post-synapse muscle tissue. These findings were surprising because she reasoned disruption in axonal transport and BDNF-TrkB signaling would result in an accumulation of mitochondrial density at the synapse and not a decrease in density. Dangremond (2016) proposed several mechanisms for the change in mitochondrial density demonstrated in msBDNF−/−. Firstly, a decrease in muscle synthesized BDNF results in decreased mitochondrial recruitment to the pre-synapse, as mitochondrial recruitment to the synapse requires BDNF-TrkB signaling.
(Dangremond, 2016; Su et al., 2014). Secondly, accumulating mitochondria may be present at
the pre-synapse but unable to sequester the mitochondrial probe (Dangremond, 2016).
Mitochondria at the pre-synapse may have a reduced electrochemical gradient and sequester less
of the MitoTracker dye, resulting in poor labeling (Dangremond, 2016).

In current studies, we aimed to reassess the role of msBDNF in maintenance of the mitochondrial
density at the neuromuscular junction by targeting the total population of mitochondria and aim
to visualize both functional mitochondria and mitochondria unable to sequester the MitoTracker
probe. To address the potential shortcomings of MitoTracker, we used immunohistochemistry to
label a structural protein of the outer mitochondrial membrane (Embacher et al., 2001; Kanaji et
al., 2000; Zhu et al., 2011). Most mitochondrial probes, such as MitoTracker, require a
membrane potential to function. Thus, dead, or damaged mitochondria may not be visible due to
a lack of membrane potential or an inability to sequester the probe. Targeting integral structural
proteins within the mitochondrial membrane allowed us to bypass the need for membrane
potential (Liu et al., 2005; Sathasivam et al., 2001).

The vast majority of mitochondrial proteins are nuclear encoded; thus, protein import is critical
for mitochondrial function. Once released into the cytosol, mitochondria targeted pre-proteins
are imported by receptors at the surface of the outer mitochondrial membrane (Di Maio et al.,
2016; Kanaji et al., 2000; Yano et al., 2004). The import of pre-proteins is heavily regulated by a
network of translocases found on the outer and inner mitochondrial membranes. Translocases of
the outer mitochondrial membrane (TOM) act as a system of checks and balances for incoming
proteins with intramitochondrial targets. For clarity, we use the abbreviation (TOM) to describe
the translocase super complex and respective subunits, and we use the abbreviation (TOMM) in
reference to a specific antibody. At the core of the TOM complex is subunit TOM40, which is
flagged by two superficial subunits TOM20 and TOM22. The superficial TOM20 subunit is an import receptor, regulating protein insertion at the outer membranes surface. The docking of both TOM20 and TOM40 to the second import receptor TOM22, facilitates the movement of preproteins into the outer mitochondrial membrane (Gold et al., 2017; Yano et al., 2004).

Given that the TOM complex is an integral structure in the outer mitochondrial membrane and regulation of import, TOM20 is an ideal mitochondrial marker to bypass membrane potential dyes. The localization of mitochondria via the TOM complex has been used in mitochondrial degeneration studies. In an immunocytochemical study of Meniere’s disease, the inner and outer hair cells were visualized by the ubiquitous distribution of TOM20 in the organ of Corti (Balaker et al., 2013). Additionally, in a recent study of early-onset Parkinson’s disease, the TOM20 mitochondrial marker was used in observation of PINK1/Parkin-dependent mitophagy (Ashrafi et al., 2014; Fiesel et al., 2017).

We hypothesized there would be a significant increase in mitochondrial populations at the neuromuscular junction in animals with decreased skeletal muscle-synthesized BDNF. We reasoned an accumulation of mitochondria in the neuromuscular junction would be due to a combination of axonal trafficking deficits, lack of BDNF-TrkB signaling, and a disruption in mitochondrial clearance. To target total mitochondrial population in the neuromuscular junction, we co-labeled mitochondria using the fluorescent probe MitoTracker™DeepRed (Molecular Probes) and a polyclonal antibody targeting the integral translocase of the outer mitochondrial membrane TOMM20 (Invitrogen™). MitoTracker™DeepRed is a fixable molecular probe which requires actively respiring mitochondria to fluoresce. TOM20 is an integral structural protein of the mitochondrial membrane and antibodies can bind to TOM20 independently of membrane potential. MitoTracker dye was injected into the gastrocnemius and endocytosed over
72 hours into the gastroc-associated motor neurons of living mice. The gastroc was then removed and sectioned post-fixation to undergo an immunohistochemical assay of TOMM20 and neuromuscular junction immunolabeling.

We were successful in visualizing mitochondrial populations within the pre-synaptic and post-synaptic compartments of skeletal muscle sections in 120d control, msBDNF<sup>+/−</sup> and msBDNF<sup>−/−</sup> mice. The pre-synaptic populations of mitochondria were defined by the colocalization of MitoTracker™DeepRed and TOMM20 to the pre-synaptic marker Synaptophysin-1. Synaptophysin is an integral transmembrane protein of synaptic vesicles and frequently used as both an axonal and synaptic marker in neurons (Chung et al., 2019; Kwon and Chapman, 2011). The post-synaptic mitochondrial populations were delineated by the colocalization of MitoTracker™DeepRed and TOMM20 to the post-synaptic marker α-bungarotoxin (Life Technologies Corporation). α-bungarotoxin is a snake venom from the Southeast Asian banded krait, <i>Bungarus multicinctus</i>, which irreversibly binds to the nicotinic acetylcholine receptors of the post-synapse (Young et al., 2003). To measure mitochondrial populations, the neuromuscular junction was remodeled using IMARIS 3D rendering software, and the colocalization of 3D voxels, representative of both mitochondrial markers, were measured in pre-synaptic and post-synaptic surfaces.
Methods:

**Muscle Injections**

The following protocol was adapted from Martin et al., (2009) and Dangremond (2016). MitoTracker™ Deep Red FM (M22426; Molecular Probes, Eugene, OR) lyophilized solid was dissolved in anhydrous dimethyl sulfoxide (DMSO) to a concentration of 1 mM stock solution. Final working solutions were prepared per diem to a final working concentration of 250 nM diluted in 0.9% saline. Animals were administered the non-steroidal anti-inflammatory drug (NSAID) Metacam® (Meloxicam, 5 mg/kg, PO, q.24.h., Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO and analgesic drug Buprenorphine (1.5 mg/kg, SC, q.6.h., Hospira, Inc., Lake Forest, IL). One hour before surgery, animals were anesthetized via Isothesia™ (Isoflurane, Henry Schein Animal Health, Dublin, OH) inhalant. A 5-mm longitudinal incision was made to the dorsal right hind limb. Intramuscular (IM) injections of the MitoTracker probe were administered at proximal, medial, and distal points along gastrocnemius muscle. Experimental animals received three IM injections, each consisting of 2 µl of MitoTracker dye and totaling in 6 µl MitoTracker injected per animal. The incision site was closed using Vetbond™ (3M Animal Care Products, St. Paul, MN) and swabbed with local anesthetic. Animal weight and behavior was closely monitored over seventy-two hours for signs of distress. Experimental animals received supplemental doses of analgesic buprenorphine and NSAID Metacam as needed.

**Muscle Harvest**

At 120-days, animals were anesthetized via Isothesia™ inhalant and then euthanized by intraperitoneal injection of Beuthanasia® D Special (Pentobarbital Sodium and Phenytoin Sodium, >290 mg/kg, IP, Merck & CO, Inc., Madison, NJ). Immediately following sacrifice, a whole animal intracardial-perfusion was performed using 0.9% saline, followed by 4% paraformaldehyde (in 1:9 0.2M monobasic:0.2M dibasic sodium phosphate buffer, pH 7.4). The
gastrocnemius muscle was removed, transferred to a 16-hour post-fix in 4% paraformaldehyde, and then immersed in 20% sucrose (in 1:9 0.2M monobasic:0.2M dibasic sodium phosphate buffer, pH 7.4) solution for 24 hours.

**Tissue Sectioning and Immunohistochemistry**

Following sucrose equilibration, the gastroc tissue was embedded in Tissue-Tek® OCT compound (Sakura® Finetek USA, Inc. Torrance, CA), and flash frozen over dry ice for preservation. Muscle tissue was longitudinally sectioned at 40 µm at -20°C, using a Leica CM1850 Cryostat and cross-linked to gelatin coated slides. Frozen tissue was protected from light and stored at -80°C until immunohistochemistry.

Tissue sections were incubated at room temperature for 1 hour with blocking buffer solution containing 10% normal donkey serum (NDS), 0.2% Triton®X-100, 0.01% sodium azide in 1XPBS. Slides were incubated in blocking solution containing primary antibodies Synaptophysin-1 (1:500, guinea pig polyclonal antibody, 101 004; Synaptic Systems, Göttingen, Germany) and TOMM20 (1:500, rabbit polyclonal antibody, PA5-52843; Invitrogen™, Carlsbad, CA) for 24 hours at 4°C. Prior to secondary antibody application, slides were rinsed in a series of 1X PBS washes (3x5min). Tissue were incubated with secondary antibodies in blocking solution: DyLight™ 405 (1:100, donkey anti-guinea pig, 706-475-148; Jackson ImmunoResearch Inc.; West Grove, PA), Alexa Fluor® 488 (1:100, donkey anti-rabbit, A-21206; Life Technologies Corporation, Eugene, OR.), and α-Bungarotoxin conjugate Alexa Fluor® 555 (1:1000, B35451; Life Technologies Corporation, Eugene, OR) for 1 hour at room temperature. Slides were rinsed with 1X PBS (3x5min) and cover slipped with ProLong™ Gold Antifade Mountant (P36930; Invitrogen™, Carlsbad, CA) to prevent fading.
Imaging and data collection

An Olympus Fluoview FV1000 confocal laser scanning microscope was used to collect images of the neuromuscular junction of gastroc-associated motorneurons. Images of gastrocnemius tissue included 40 junctions per animal per experimental group. Gastroc imaging parameters were set as follows: 20 μs/pixel scanning speed, 1024 x 1024 aspect ratio, 2.5 digital zoom and 4.77μm step size. Confocal images were uploaded to IMARIS® 3D rendering software (Bitplane Scientific Software). Individual surfaces were created to delineate the neuromuscular junction by immunolocalization of pre-synaptic synaptophysin, post-synaptic acetylcholine receptors, protein TOMM20 and MitoTracker dye. Four additional surfaces were created to measure the colocalization within the neuromuscular junction; (1) Synaptophysin to TOMM20, (2) Acetylcholine receptors to TOMM20, (3) Synaptophysin to MitoTracker™Deep Red, and (4) Acetylcholine receptors to MitoTracker™Deep Red. Mitochondrial density at the pre and post synapse was measured and compared using a one-way ANOVA.
**Results:**

Previous work demonstrated a significant decrease in overlapping voxels at the pre-synapse between MitoTracker™ and VAcT in Muscle\textsuperscript{BDNF/-} mice, while there was no significant change in overlapping voxels at the post-synapse (Dangremond, 2016). To quantify mitochondrial populations within the pre- and post-synapse of the gastroc associated motorneurons, the average number of overlapping voxels was measured for each colocalization surface created using IMARIS® 3D rendering software. There was no significant change in mean voxel colocalization across all genotypes in both pre- and post-synaptic surfaces at 120 days of age.

![Figure 17](image)

Figure 17. Photomicrographs revealed pre-synaptic (A) synaptophysin and post-synaptic (B) α-bungarotoxin immunolabeling. Localization of mitochondrial densities are revealed by (C) MitoTracker™ dye and outer mitochondrial membrane protein (D) TOMM20 immunolabeling. Merging of immunolabeled surfaces (E) delineate the neuromuscular junction. Scale bar = 30 µm.
Figure 18. (A) IMARIS 3D rendered photomicrograph used to measure the colocalization of pre-synaptic surface Synaptophysin (blue) and MitoTracker™Deep Red (red) voxels. (B) Mean voxels of MitoTracker™Deep Red and Synaptophysin colocalization in the pre-synaptic terminal of control and muscle-synthesized BDNF deficient mice at 120d. There was no significant difference in mean voxel colocalization of MitoTracker™Deep Red and Synaptophysin in the pre-synapse among genotypes at 120d (N=8; F=1.21; df=2,21; P=0.32).

Figure 19. (A) IMARIS 3D rendered photomicrograph used to measure the colocalization of post-synaptic surface α-bungarotoxin (yellow) and MitoTracker™Deep Red (red) voxels. (B) Mean voxels of MitoTracker™Deep Red and α-bungarotoxin colocalization in the post-synaptic terminal of control and muscle-synthesized BDNF deficient mice at 120d. There was no significant difference in mean voxel colocalization of MitoTracker™Deep Red and α-bungarotoxin in the post-synapse among genotypes at 120d (N=8,7,7; F=0.15; df=2,19; P=0.85).
Figure 20. (A) IMARIS 3D rendered photomicrograph used to measure the colocalization of pre-synaptic surface Synaptophysin (blue) and TOMM20 (green) voxels. (B) Mean voxels of TOMM20 and Synaptophysin colocalization in the pre-synaptic terminal of control and muscle-synthesized BDNF deficient mice at 120d. There was no significant difference in mean voxel colocalization of TOMM20 and Synaptophysin in the pre-synapse among genotypes at 120d (N=8; F=1.00; df=2,21; P=0.38).

Figure 21. (A) IMARIS 3D rendered photomicrograph used to measure the colocalization of post-synaptic surface α-bungarotoxin (yellow) and TOMM20 (green) voxels. (B) Mean voxels of TOMM20 and α-bungarotoxin colocalization in the post-synaptic terminal of control and muscle-synthesized BDNF deficient mice at 120d. There was no significant difference in mean voxel colocalization of TOMM20 and α-bungarotoxin in the post-synapse among genotypes at 120d (N=8; F=1.12; df=2,21; P=0.34).
Discussion:

Mitochondrial labeling is not significantly altered in the pre-synaptic or post-synaptic terminals of msBDNF deficient mice compared to controls at 120 days of age. A trend of increasing immunolabeling at the pre- and post-synapse is present and suggest an increase in mitochondrial population within the neuromuscular junction of msBDNF deficient mice when compared to controls. An increasing trend of TOMM20 immunolabeling in the pre-synaptic and post-synaptic compartments is demonstrated in both msBDNF+/− and msBDNF−/− deficient mice, while the MitoTracker dye demonstrates an increasing population of mitochondria in the pre-synapse of msBDNF−/− mice. This trend suggest that mitochondria are accumulating in the neuromuscular junction under conditions of deficient msBDNF.

These data are inconsistent with previous findings which demonstrated MitoTracker™ labeling was significantly decreased in the pre-synaptic boutons of msBDNF−/− animals at 120 days of age (Dangremond, 2016). However, in a previous study, Dangremond (2016) theorized that mitochondrial labeling may be incomplete and dead or degenerating mitochondria were not able to sequester the dye due to a lack of electrochemical gradient. Our current data supports this hypothesis, and the use of a co-labeling system to target the total mitochondrial population versus only those with an active membrane potential. The use of an antibody binding to TOM20 in combination with the MitoTracker dye may enable both active and inactive mitochondria to be labeled within the motor unit. These findings may demonstrate a trend of accumulating mitochondria within the pre-synapse of msBDNF deficient mice. Further, mitochondrial accumulation may be a byproduct of disrupted mitochondrial dynamics due to a loss in BDNF-TrkB mediated signaling. Additionally, our statistical assessment of the current study demonstrates significant variability in our heterozygous and homozygous msBDNF deficient...
mice when compared to controls. Variance appears equal among control groups throughout the assessment of the neuromuscular junction. The increasing levels of variance visible in heterozygous and homozygous knockout groups suggest the current study may benefit from a larger sample size.

Our current study provides a baseline measurement for mitochondrial density at the pre-synapse, but also illuminates the need for continued research. Studies have shown that mitochondria are transported via anterograde and retrograde motor protein complexes, and BDNF signaling supports both transport and docking of mitochondria at the pre-synapse (Huang and Reichardt, 2003; Su et al., 2014). However, the signaling pathways supporting mitochondrial transport within the motor neuron are not well understood. Our study demonstrates an increasing trend of mitochondrial density at the pre-synapse in msBDNF deficient mice (Figure 18, Figure 20). The accumulation of mitochondria within the pre-synapse may be the result of increased anterograde transport or decreased retrograde transport. BDNF-TrkB binding arrest mitochondrial motility by increasing intracellular Ca$^{2+}$ and the activation of the PI3K pathway, and a loss of BDNF increases mitochondrial motility in kinesin-Miro1 linked anterograde complexes. The molecular pathways promoting retrograde transport of mitochondria are still largely unknown.

Interestingly, the pre-synaptic trend of increasing mitochondrial populations suggest both actively respiring mitochondria and dysfunctional mitochondria are accumulating. It is possible msBDNF-TrkB binding supports signaling pathways regulating mitochondrial dynamics such as fission, fusion, and mitophagy. A lack of msBDNF may alter mitochondrial dynamics. BDNF-TrkB signaling activates several pathways that increase calcium levels locally, and changes to calcium levels may affect calcium sensing adaptor proteins such as Miro1 or the recruitment of adaptors such as fission protein Drp1. Further BDNF-TrkB signaling has been shown to be
antiapoptotic. Lack of msBDNF-TrkB signaling may alter mitochondrial clearance through BNIP3 mitophagy pathways described in Chapter 1 and result in increasing accumulation of dysfunctional mitochondria at the pre-synapse.

Our findings suggest transport of mitochondria, mitochondrial dynamics or both are altered in msBDNF deficient mice. How and if msBDNF expression alters mitochondrial dynamics directly has yet to be explored. Future studies should focus specifically on adaptor proteins involved in mitophagy, transport, fission, and fusion. These studies should aim to demonstrate how msBDNF expression may alter mitochondrial dynamics through adaptor protein activation locally. Targeting adaptor proteins such as Miro1 would help elaborate the possible role for msBDNF signaling in transport, docking and mitochondrial clearance. Further, future studies should aim to determine if accumulation of mitochondria in the pre-synapse is due to an increase in anterograde transport or lack of retrograde transport due to msBDNF expression.
CHAPTER FOUR: ASSESSMENT OF MITOCHONDRIAL DYSFUNCTION IN THE LIGATED SCIATIC NERVE OF SKELETAL MUSCLE-SYNTHESIZED BDNF DEFICIENT MICE

Introduction:

Much like the synaptic terminal, mitochondria are densely populated throughout the axon due to the high energy demands of transport. The axon works as a transportation network comprised of vesicles, motor complexes, adaptor proteins, microtubules, actin, and neurofilaments. Mitochondria are transported anterogradely from the cell soma to the axon terminal via motor complexes, and dysfunctional mitochondria are transported retrogradely back to the soma for recycling. Axonal transport works to support the neuron from development to death, and a multitude of molecular mechanisms require axonal transport for optimal communication throughout the elaborate architecture of a neuron. Newly synthesized proteins and lipids are transported peripherally from the soma, while damaged proteins are recycled back to the soma. However, axonal transport itself requires functional mitochondria. Mitochondria are not only transported along the axon, but also work to fuel the transportation machinery.

Mitochondrial transport along the axon is a unique process in itself. Mitochondria are transported bidirectionally, along microtubules and actin, undergo frequent docking, change their velocity, and have been shown to switch directions. Unsurprisingly, disruption in mitochondrial transport has been heavily characterized in diseases within the nervous system. However, if mitochondrial dysfunction is a result of disruption in transport or due to the accumulation of faulty mitochondria lacking clearance is still unclear. As previously described in Chapter 1, microtubule-based transport of mitochondria is largely dependent on kinesin motor complexes in
the anterograde direction, however retrograde transport of mitochondria is significantly less understood. In general, retrograde transport along the axon requires both the dynein motor complex and DCTN1 adaptor protein. Although little is known about mitochondrial transport in the retrograde direction, DCTN1 is one of the few adaptor proteins shown to localize with the mitochondrial membrane.

Recent studies have indicated a possible role for msBDNF/TrkB receptor signaling as a regulator of DCTN1 mediated retrograde transport. In a study aimed at characterizing dendritic morphology, experimental animals were injected with the retrograde tracer Fluorogold, and a significant decrease in dendrite length was found in msBDNF+/+ and msBDNF−/− mice (Pomeroy, 2013). It was reasoned the observed loss of dendritic length was due to disruption to retrograde transport along the axon altering immunolabeling at the proximally located dendrites (Pomeroy, 2013). In a subsequent study of retrograde transport in msBDNF deficient mice, the sciatic nerve was ligated to create a physical obstruction to axonal transport. Using an immunohistochemical assay, the accumulation of bi-directionally transported synaptophysin, NF-H-P, and retrograde transport adaptor protein DCTN1 were measured at the proximal and distal points of ligation (Dangremond, 2016). The assessment of DCNT1 accumulation distal from the point of ligation demonstrated a significant decrease in DCNT1 and NF-H-P immunolabeling at 120 days of age in msBDNF−/− mice compared to controls (Dangremond, 2016). The study provided further evidence linking msBDNF expression to a disruption in retrograde transport.

To further elucidate msBDNF/TrkB mediated transport the sciatic nerve was ligated and immunohistochemical assay was used to measure active phosphorylated TrkB receptors along the axon. A significant difference in mean immunofluorescence of phosphorylated TrkB was found in msBDNF+/− and msBDNF−/− mice at 120d distal to the point of ligation compared to
controls (VanOsdol, 2018). Further, the assessment of DCTN1 accumulation distal to the point of ligation demonstrated a significant decrease in DCTN1 immunolabeling compared to controls at 120d (VanOsdol, 2018). Together, these findings support a mechanism for msBDNF/TrkB-mediated signaling in the recruitment of DCTN1 to retrograde transport dynein motor complexes. BDNF regulates surface expression of TrkB receptors, and loss of msBDNF disrupts endosomal recruitment of TrkB receptors to DCTN1-dynein motor complexes (VanOsdol, 2018).

Due to the deficits in retrograde transport demonstrated in msBDNF deficient mice, the present study aims to assess the role of msBDNF in the transport of mitochondrial populations along the motor neuron axon. Chapter three describes mitochondrial populations of msBDNF deficient mice at 120d in the gastroc-associated neuromuscular junction, where an increasing trend of accumulating mitochondria was found, but did not reach significance. We reason that accumulating mitochondria within the pre-synapse would promote increased axonal transport bi-directionally. Increased mitochondrial populations within the pre-synapse may indicate an increase in the anterograde transport of mitochondria, while simultaneously requiring an increase in the retrograde transport of mitochondria targeted for recycling.

In order to determine if mitochondrial populations are significantly altered due to potential transport deficits in msBDNF deficient mice, animals underwent a sciatic nerve ligation protocol at 120d. Originating within the lower spinal cord, the sciatic nerve is large bundle of mixed nerves which innervate the lower limbs. Ligating the sciatic nerve disrupts the bidirectional transport along motor neuron axons by creating a physical barrier. The gastrocnemius muscle was injected with the mitochondrial probe MitoTracker™ Deep Red 72 hours prior to ligation, which then enabled the MitoTracker probe to diffuse into the gastroc-associated motor neurons
and be sequestered by actively respiring mitochondria prior to ligation. The sciatic nerve was ligated for eight hours, and harvested post-fixation for immunohistochemical assay. Immunohistochemical assay techniques were used to label the prominent structural protein TOMM20, synaptic vesicle marker Synaptophysin and retrograde transport adaptor protein DCTN1. Mitochondrial populations within the motor axon were assessed by measuring the accumulation of MitoTracker™Deep Red, TOMM20, Synaptophysin and DCTN1 at both proximal and distal points of ligation. Under conditions of normal axonal transport, we would expect increasing accumulation of immunolabels at the ligation barrier as transport has physically been disrupted. We hypothesized that due to axonal retrograde transport deficits, a resulting decrease in mitochondrial accumulation would be found distal to the point of ligation in msBDNF deficient mice compared to controls.

We were successful in visualizing mitochondrial populations along the axon of 120d control, msBDNF+/− and msBDNF−/− mice. The axonal populations of mitochondria were defined by the mean voxel immunofluorescence of the MitoTracker™Deep Red (Molecular Probes) and TOMM20 (Invitrogen™) both proximally and distally from the point of ligation. Synaptophysin-1 (Synaptic Systems) is transported in both anterograde and retrograde directions and mean voxel immunofluorescence was used as internal control for the ligation study. Possible deficits in retrograde axonal transport were determined by mean voxel immunofluorescence of DCTN1 (Abcam) and examined both proximally and distally from the point of ligation. As DCTN1 is one of the few retrograde adaptor proteins known to localize with the mitochondrial membrane, the colocalization of MitoTracker™DeepRed and TOMM20 to DCTN1 was used to examine retrograde transport of mitochondria specifically on either side of the ligation. To assess mitochondrial populations and axonal transport, the axon was analyzed using IMARIS 3D
rendering software and 3D voxel representation of immunolabels were measured proximally and distally from the point of ligation.

**Methods:**

*Muscle Injections*

The following protocol was adapted from Martin et al. (2009) and Dangremond (2016). MitoTracker® Deep Red FM (M22426; Molecular Probes, Eugene, OR) lyophilized solid was dissolved in anhydrous dimethyl sulfoxide (DMSO) to a concentration of 1 mM stock solution. Final working solutions were prepared per diem to a final working concentration of 250 nM diluted in 0.9% saline. Animals were administered the non-steroidal anti-inflammatory drug (NSAID) Metacam® (Meloxicam, 2.5 mg/kg, PO, q.24.h., Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO and analgesic drug Buprenorphine (1.5 mg/kg, SC, q.6.h., Hospira, Inc., Lake Forest, IL). One hour before surgery, animals were anesthetized via Isothesia™ (Isoflurane, Henry Schein Animal Health, Dublin, OH) inhalant. A 5-mm longitudinal incision was made to the dorsal right hind limb. Intramuscular (IM) injections of the MitoTracker probe were administered at proximal, medial, and distal points along gastrocnemius muscle. Experimental animals received three IM injections, each consisting of 2 µl of MitoTracker dye and totaling in 6 µl MitoTracker injected per animal. The incision site was closed using Vetbond™ (3M Animal Care Products, St. Paul, MN) and swabbed with local anesthetic. Animal weight and behavior was closely monitored over seventy-two hours for signs of distress. Experimental animals received supplemental doses of analgesic buprenorphine (1.5 mg/kg, SC, q.6.h.) and NSAID Metacam (2.5 mg/kg, PO, q.24.h) as needed.
Sciatic Nerve Ligation

The protocol used for sciatic nerve ligations in experimental animals is an adaptation from Katsuno et al. (2006) and Dangremond (2016) protocols. One hour prior to surgery, animals were administered the non-steroidal anti-inflammatory drug Metacam® (5 mg/kg, PO) and the analgesic drug buprenorphine (2.5 mg/kg, SC, q.8). Animals were anesthetized via Isothexia™ inhalant. Anesthetic plane was monitored closely and adjusted according to animal behavior throughout the procedure. The dorsal right hindlimb was gently palpated at mid-thigh to orient the femurs location. The sciatic nerve is located just caudal to the femur and lays deep to the musculature. Proper anatomical orientation is critical for limiting post-operative pain and distress in the animal. A small orthogonal incision was made superficial to the nerve, and the musculature was palpated until the sciatic nerve was exposed. The sciatic nerve was ligated using 7.0 surgical grade silk suture. The wound was closed using a 7-mm stainless steel wound clip and swabbed with local anesthetic.

Animals were monitored for signs of distress every thirty minutes, over the course of eight hours. Six hours post-ligation, animals received a supplemental analgesic dose of buprenorphine (2.5 mg/kg, SC). Eight hours post-ligation, animals were anesthetized via Isothexia™ inhalant and euthanized via Beuthanasia®D Special (Pentobarbital Sodium and Phenytoin Sodium, >290 mg/kg, IP, Merck & CO, Inc., Madison, NJ). Immediately following sacrifice, a whole animal intracardial-perfusion was performed using 0.9% saline, followed by 4% paraformaldehyde (in 1:9 0.2M monobasic:0.2M dibasic sodium phosphate buffer, pH 7.4). The ligated nerve was re-exposed, and an additional ligation was tied at the most distal end of nerve for orientation. The maximum length of exposed nerve was harvested, leaving a minimum of 5 mm of tissue at both proximal and distal ends of the central ligation knot. The extracted nerve underwent a 1-hour
post-fixation in 4% paraformaldehyde, followed by 20% sucrose (in 1:9 0.2M monobasic:0.2M dibasic sodium phosphate buffer, pH 7.4) immersion for 24 hours.

**Tissue Sectioning and Immunohistochemistry**

Following sucrose equilibration, the sciatic nerve was embedded in Tissue-Tek® OCT compound (Sakura® Finetek USA, Inc. Torrance, CA) and flash frozen over dry ice prior to nerve sectioning. The sciatic nerve was longitudinally sectioned at 14 µm in -20°C using a Leica CM 1850 Cryostat and cross-linked to gelatin coated slides. Frozen nerve tissue was protected from light and stored at -20°C until immunolabeling.

Tissue sections were incubated at room temperature for 1 hour with a blocking solution containing 10% NDS, 0.2% Triton®X-100, 0.01% sodium azide in 1X PBS. Tissues were incubated for 24 hours at 4°C with the following primary antibodies in blocking solution: Anti-Synaptophysin-1 (1:500, guinea pig polyclonal antibody, 101 004; Synaptic Systems, Göttingen, Germany), Anti-DCTN1 (1:500, goat polyclonal antibody, ab11806; Abcam; Cambridge, United Kingdom), and Anti-Tom20 (F-10) (1:500, rabbit polyclonal antibody, PA5-52843; Invitrogen™, Carlsbad, CA) in blocking solution. Following primary incubation, the slides were rinsed in a series of 1X PBS washes (3x5min) prior to secondary antibody application. Tissues were incubated at room temperature for 1 hour with the following secondary antibodies in blocking solution: DyLight™ 405 (1:100, donkey anti-guinea pig, 706-475-148; Jackson ImmunoResearch Inc.; West Grove, PA), Alexa Fluor® 488 (1:100, donkey anti-rabbit, A-21206; Life Technologies Corporation, Eugene, OR.), and Rhodamine (TRITC) (1:100, donkey anti-goat, 705-025-147; Jackson ImmunoResearch Inc.; West Grove, PA). Sections were rinsed
with 1XPBS (3x5min) and cover slipped with ProLong™ Gold Antifade Mountant (P36930; Invitrogen™, Carlsbad, CA) to prevent fading.

**Imaging and data collection**

An Olympus Fluoview FV1000 confocal laser scanning microscope was used to collect images of the ligated sciatic nerves. Images of the sciatic nerve totaled in 10 images per animal, per experimental group: 5 of the images were proximal to the ligation site, and 5 of the images were distal to the ligation site. Sciatic nerve imaging parameters were set as follows: 20 µs/pixel scanning speed, 1040 x 1040 aspect ratio, 1.6 digital zoom, and 0.44 µm step size. Confocal images will be uploaded to IMARIS® 3D rendering software. The total surface area of Anti-Synaptophysin-1, Anti-DCTN1, Anti-Tom20 and MitoTracker™Deep Red 633 immunolabeling was measured 500 µm from the point of ligation. The accumulation of proteins both proximal and distal from the point of ligation was measured and tested for significance using a one-way ANOVA statistical analysis.
Results:

A physical barrier in axonal transport was created using sciatic nerve ligations in msBDNF deficient mice and controls at 120d. Mean voxel immunofluorescence of Synaptophysin, DCTN1, TOMM20 and MitoTracker™DeepRed was measured at both the proximal and distal points of ligation across all genotypes. Additionally, colocalization of MitoTracker™DeepRed and TOMM20 to molecular adaptor protein DCTN1 was measured to determine possible deficits in retrograde transport of mitochondria in control, msBDNF+/- and msBDNF-/- mice.

At 120d, there was no significant change in mean voxel immunofluorescence immediately distal and proximal to the point of ligation in the internal control synaptophysin. Mean voxel immunofluorescence of the retrograde adaptor protein DCTN1 immediately distal and proximal to the point of ligation had no significant change across all genotypes. Mitochondrial populations defined by MitoTracker™DeepRed and TOMM20 3D voxels mean immunofluorescence demonstrated no significant difference at both proximal and distal locations across all genotypes. Finally, no significant change was found in the mean immunofluorescence for 3D voxel colocalization of DCTN1 to MitoTracker™DeepRed and TOMM20 across all genotypes.
Figure 22. Representative photomicrographs of sciatic nerve immunolabeling and accumulation immediately distal to the point of ligation. Distal accumulation of (A) Dynactin, (B) Tom20, (C) MitoTracker™ and (D) synaptophysin immunofluorescence was measured 500 μm from the point of ligation. Merging of immunolabeled surfaces (E) delineates distal nerve accumulation. Scale bar = 120 μm.
Figure 23. IMARIS 3D construct of the immunolabeled proximal ligation point along the sciatic nerve. (A) Surface localization of Synaptophysin in cyan. (B) Surface localization of DCTN1 in magenta. (C) Surface localization of MitoTracker™Deep Red in red. (D) Surface localization of TOMM20 in green. (E) Merged surface construct of immunolabeled sciatic nerve with surface channels Synaptophysin, DCTN1, TOMM20 and MitoTracker™Deep Red. Scale Bar = 100µm

Figure 24. Mean voxels of Synaptophysin accumulation immediately proximal (A) and distal (B) at the point of ligation in 120d control and muscle-synthesized BDNF deficient mice. There was no significant difference in mean voxel accumulation of Synaptophysin at the proximal ligation point among genotypes at 120d (N=8; F=2.03; df=2,21; P=0.15). There was no significant difference in mean voxel accumulation of Synaptophysin at the distal ligation point among genotypes at 120d (N=8; F=0.06; df=2,21; P=0.94).
Figure 25. Mean voxels of DCTN1 accumulation immediately proximal (A) and distal (B) at the point of ligation in 120d control and muscle-synthesized BDNF deficient mice. There was no significant difference in mean voxel accumulation of DCTN1 at the proximal ligation point among genotypes at 120d (N=8; F=0.09; df=2,21; P=0.91). There was no significant difference in mean voxel accumulation of DCTN1 at the distal ligation point among genotypes at 120d (N=8; F=1.54; df=2,21; P=0.23).

Figure 26. Mean voxels of MitoTracker™Deep Red accumulation immediately proximal (A) and distal (B) at the point of ligation in 120d control and muscle-synthesized BDNF deficient mice. There was no significant difference in mean voxel accumulation of MitoTracker™Deep Red at the proximal ligation point among genotypes at 120d (N=8; F=0.65; df=2,21; P=0.53). There was no significant difference in mean voxel accumulation of MitoTracker™Deep Red at the distal ligation point among genotypes at 120d (N=8,8,7; F=0.02; df=2,20; P=0.97).
Figure 27. Mean voxels of TOMM20 accumulation immediately proximal (A) and distal (B) at the point of ligation in 120d control and muscle-synthesized BDNF deficient mice. There was no significant difference in mean voxel accumulation of TOMM20 at the proximal ligation point among genotypes at 120d (N=8; F=1.06; df=2,21; P=0.36). There was no significant difference in mean voxel accumulation of TOMM20 at the distal ligation point among genotypes at 120d (N=8,7,8; F=1.91; df=2,20; P=0.17).

Figure 28. Mean voxels of MitoTracker™Deep Red and DCTN1 colocalized accumulation immediately proximal (A) and distal (B) at the point of ligation in 120d control and muscle-synthesized BDNF deficient mice. There was no significant difference in mean voxel colocalization of MitoTracker™Deep Red and DCTN1 proximal to the point of ligation among genotypes at 120d (N=8,7,8; F=0.02; df=2,20; P=0.98). There was no significant difference in mean voxel colocalization of MitoTracker™Deep Red and DCTN1 distal to the point of ligation among genotypes at 120d (N=8; F=0.52; df=2,21; P=0.61).
Figure 29. Mean voxels of TOMM20 and DCTN1 colocalized accumulation immediately proximal (A) and distal (B) at the point of ligation in 120d control and muscle-synthesized BDNF deficient mice. There was no significant difference in mean voxel colocalization of TOMM20 and DCTN1 proximal to the point of ligation among genotypes at 120d (N=8; F=0.42; df=2,21; P=0.66). There was no significant difference in mean voxel colocalization of TOMM20 and DCTN1 distal to the point of ligation among genotypes at 120d (N=8; F=0.37; df=2,21; P=0.69).
Discussion:

At 120d, there was no significant change in accumulation of synaptophysin, DCTN1, MitoTracker™DeepRed, or TOMM20 distal to the point of ligation across all genotypes. However, we did see a decreasing trend of TOMM20 accumulation at both proximal and distal points of ligation in msBDNF deficient mice, although this trend does not reach significance (Figure 27). Although we found no evidence of significant deficits to axonal transport, our findings do bring to light several key points supporting further discussion and study.

First, we did not find evidence supporting disrupted retrograde transport in msBDNF deficient mice. These findings are inconsistent with previous studies which showed a significant decrease in DCTN1 accumulation at the distal ligation point in msBDNF deficient mice at 120d when compared to controls (Dangremond, 2016; VanOsdol, 2018). Secondly, the trend of accumulating mitochondria in the neuromuscular junction does not appear to be the result of disrupted axonal transport. Once again, these findings are inconsistent with previous studies which provided evidence suggesting accumulation of NF-H-P in the distal axon was a result of disrupted retrograde transport in 120d msBDNF−/− animals (Dangremond, 2016). Finally, the trend suggesting a decrease in mitochondrial populations at the distal point of ligation does not appear to be a product of DCNT1 driven mobility. These last two points are of significant interest when considering mitochondrial transport and populations.

In Chapter 1, we extensively discussed the complexity in which mitochondrial dynamics regulate mitochondrial mobility, docking and morphology. Accumulating mitochondrial populations within the pre-synapse may not be explained solely by deficits in transport. BDNF-TrkB receptor binding has been shown to arrest mitochondrial motility at the synapse (Su et al., 2014). A loss of msBDNF-TrkB binding at the pre-synapse may promote mobility and result in increasingly
mobile mitochondria along the axon. However, this model does not explain accumulating mitochondrial populations at the pre-synapse or the trend of decreasing mitochondria populations distal the point of ligation. More than likely, the trends observed in mitochondrial populations during both studies are a product of several factors involved in mitochondrial dynamics.

Another possible explanation for the patterns observed in transport and synaptic populations is a role for msBDNF-TrkB binding in mitophagy or clearance. BDNF/TrkB receptor binding was shown to induce mitophagy through TrkB activation of the HIF-1α/BNIP3 complex on the outer mitochondrial membrane (Jin et al., 2019). A loss in msBDNF-TrkB activation may prevent the clearance of dysfunction mitochondria through mitophagy. This may explain the increasing accumulation of mitochondria in msBDNF deficient mice at the pre-synapse. Furthermore, dysfunctional mitochondria are then unable to mobilize out of the pre-synapse resulting in a trend of decreasing mitochondrial populations at the distal ligation point (Figure 27). Increasing intracellular Ca\(^{2+}\) levels due to accumulating dysfunctional mitochondria may inhibit motility driven by calcium sensing regulators, such as Miro1. Therefore, dysfunctional mitochondria are both accumulating distally and subsequently unable to mobilize.

Finally, the sciatic nerve ligation protocol gave us a unique opportunity to further investigate the trend of decreasing mitochondrial populations at the distal point of ligation, and so we further characterized mitochondrial transport driven by the adaptor protein DCNT1 using additional statistical testing (Figure 28Figure 29). As previously discussed, DCTN1 is one of the few adaptor proteins shown to localize with mitochondria moving in the retrograde direction. In fact, very few studies have been able to characterize the molecular complexes that drive mitochondria back to the soma. We aimed to determine if mitochondrial populations colocalized to DCTN1 were accumulating at both ligation points, and further elucidate trending mitochondrial
populations driven by DCTN1 on the axon. We found no significant difference in DCNT1 accumulation distal to the point of ligation but did see a declining trend in mitochondrial populations distal to the point of ligation (Figure 27). However, there was no significant difference in mitochondrial colocalization to DCTN1 both proximally and distally from the point of ligation across all genotypes (Figure 28Figure 29). These findings are of significant interest and may suggest that retrograde transport of mitochondria is not solely dependent on the DCTN1-dynein adaptor motor complex. More than likely, unknown mediators of transport are present and working to transport mitochondrial populations simultaneously.
CHAPTER FIVE: SUMMARY AND CONCLUSIONS

“Mitochondrial dynamics” is the all-inclusive term used to describe the molecular pathways in which mitochondria promote the maintenance and survival of a cell. Mitochondrial dynamics are of significant importance in the neuron and not only work to meet energy demands, but promote axonal transport, synaptic docking, synaptic plasticity, and apoptotic events. Although research has expanded on our understanding of mitochondrial dynamics over the past twenty years, much is still unknown about the intricate molecular pathways that facilitate the maintenance of mitochondria. One possible regulator of mitochondrial mechanics are neurotrophic growth factors. Neurotrophic factors support neuron development, health, and survival, and so a relationship between the equally supportive organelle and neurotrophic factor expression is not surprising. In fact, neurotrophic factors support several of the same mechanism critical to neuron survival, such as axonal transport, synaptic plasticity, development, and cell death.

Previous studies in our lab have characterized the role of muscle-synthesized BDNF in the maintenance of the motor unit. Histological analysis of skeletal muscle deficient in muscle-synthesized BDNF demonstrates severe morphological changes to tissue and lack of msBDNF was shown to induce myopathy (Dangremond, 2016). Interestingly, expression of muscle-synthesized BDNF appears to play an essential role in the maintenance of both skeletal muscle and the innervating neuron. Previous findings in our laboratory have demonstrated that a lack of skeletal muscle-synthesized BDNF results in a reduced dendritic outgrowth, increasing synaptic fragmentation, and deficits in axonal transport in the innervating motor neuron (Dangremond, 2016; Pomeroy, 2013; VanOsdol, 2018). We reasoned that deficits in axonal transport at 120d,
induced by a lack of msBDNF expression, may alter mitochondrial populations in the motor neuron. Mitochondrial dysfunction is heavily cited throughout neurodegenerative and neuromuscular diseases such as Amyotrophic Lateral Sclerosis, Spastic Paraplegia, Charcot-Marie-Tooth, Alzheimer’s disease, Huntington’s disease and Parkinson’s disease (Borgia et al., 2017; Chen and Chan, 2009; Chevalier-Larsen and Holzbaur, 2006; Correia and Moreira, 2018; Cozzolino and Carrì, 2012; Jiang et al., 2015; Sasaki and Iwata, 2007; Saxton and Hollenbeck, 2012; Stavoe and Holzbaur, 2018). A disruption to axonal transport, synaptic docking, or mitochondrial clearance mechanisms may explain the adult onset of pathophysiology characterized in msBDNF deficient mice at 120d. We hypothesized that reduced msBDNF would result in disrupted axonal transport of mitochondria, and subsequently increase mitochondrial populations at the neuromuscular junction.

To characterize the role of msBDNF in the maintenance of mitochondrial density, experimental animals were injected with a mitochondrial probe which freely diffused into the gastrocnemius associated motor neurons. Dangremond (2016) found significantly decreased labeling of mitochondria in msBDNF−/− knockouts at the axon terminal and a declining trend in msBDNF+/− knockouts (Figure 11). Additionally, a trend of decreasing mitochondrial density was found in the post-synapse of MuscleBDNF+/− animals when compared to controls (Figure 11; Dangremond, 2016). However, the study was limited by the MitoTracker probe used to label density, as the probe is only sequestered by functional mitochondria. Therefore, potentially dysfunctional mitochondria may be accumulating at the axonal terminal and unable to sequester the MitoTracker dye. To expand on our understanding of mitochondrial populations in the absence of msBDNF we designed a co-labeling protocol which combined the intramuscular injections of a mitochondrial probe with an additional immunohistochemical assay targeting an
integral structural protein, TOMM20. The use of a co-labeling protocol enabled us to visualize actively respiring mitochondria, while simultaneously targeting mitochondrial populations independently of membrane potential.

The implementation of co-labeling protocol was successful and resulted in the visualization of total mitochondrial populations at both the neuromuscular junction and along the axon. When assessing mitochondrial populations at the neuromuscular junction, we found no significant difference in mitochondrial marker colocalization within the pre- and post-synaptic surfaces at 120d, across all genotypes (Figure 18B, 19B, Figure 20B, and 21B). However, our findings demonstrate an increasing trend of TOMM20 accumulation in both heterozygous and homozygous knockouts, and an increasing trend in accumulation of the MitoTracker probe in homozygous knockouts within the pre-synaptic surface at 120d (Figure 18B, Figure 20B). Although neither trend reaches significance, it does suggest mitochondrial populations may be accumulating within the pre-synaptic surfaces under conditions of deficient msBDNF. When assessing mitochondrial populations along the axons of the sciatic nerve, we found no significant accumulation in mitochondrial markers both proximal and distal from the point of ligation (Figure 26B, Figure 27). We do see a decreasing trend overall in TOMM20 accumulation both distal and proximal to the point of ligation, however this trend does not reach significance (Figure 27). These findings suggest transport of mitochondrial populations is disrupted in mice lacking muscle-synthesized BDNF. Through further statistical analysis, we also demonstrate that the trend of decreasing TOMM20 accumulation at ligation points appear to be independent of DCTN1 colocalization at proximal and distal regions (Figure 29).

Both experiments aimed to demonstrate mitochondrial populations within the motorneurons of msBDNF deficient mice. Our findings suggest that mitochondrial populations may be
accumulating in the pre and post-synapse of msBDNF deficient mice, although this pattern of accumulation did not reach significance. Further, mitochondrial transport does not appear to be disrupted msBDNF deficient mice. However, both studies of mitochondrial populations fail to consider mitochondrial dynamics. These findings ultimately provide us a baseline measurement of mitochondrial populations within the motor neuron, and nothing more. This point is of significant importance and requires further investigation and study for several reasons. The molecular pathways that maintain mitochondrial dynamics must be targeted specifically in order to elucidate how and if muscle-synthesized BDNF-TrkB receptor binding alters mitochondrial populations. An accumulation of mitochondria in the neuromuscular junction could be the result of several factors and expanding our understanding of mitochondrial populations will require the targeting of specific adaptor proteins which regulate transport, docking, and mitophagy.

Further, mitochondrial transport is both slow and fast acting, undergoes frequent docking, and has been shown to change direction. To expand on our understanding of axonal transport of mitochondrial populations, future studies must target specific modes and regulators of transport along the axon. Lastly, our current study fails to recognize changes to mitochondrial morphology. Mitochondrial morphology is of upmost importance when considering the degree of dysfunction and continues to be a limitation in most studies of mitochondrial dynamics. Mitochondria undergo morphological changes to ultrastructure under conditions of dysfunction. Mitochondrial populations often fuse, change shape and size, fragment or elongate into larger super structure under conditions of stress. Changes to ultrastructure are a better indicator of dysfunction and damage, but extremely difficult to track.

Our current study is limited by several factors, but future studies aimed to target specific molecular pathways would significantly expand on our understanding of mitochondrial dynamics.
and neurotrophic support. Mitochondrial accumulation in the neuromuscular junction may be a byproduct of disrupted mitochondrial dynamics due to a loss in BDNF-TrkB mediated signaling locally. Future studies should aim to investigate if msBDNF-TrkB receptor activation supports signaling pathways regulating mitochondrial dynamics such as fission, fusion, and mitophagy. It is possible that BDNF-TrkB signaling activates several pathways that increase calcium levels locally, and changes to calcium levels subsequently effect calcium sensing adaptor proteins such as Miro1. Further, future studies should aim to assess the antiapoptotic effects of BDNF-TrkB receptor activation in the axon terminal. Lack of msBDNF-TrkB signaling may alter mitochondrial clearance through mitophagy pathways such as BNIP3 and result in increasing accumulation of dysfunctional mitochondria at the pre-synapse. Future studies should focus specifically on adaptor proteins involved in mitophagy, fission, and fusion, and aim to demonstrate how msBDNF expression may alter mitochondrial dynamics through adaptor protein activation. The results presented in this thesis elaborate on the complexity of mitochondrial dynamics and demonstrate the importance of further investigation into neurotrophic support of mitochondrial maintenance.
REFERENCES


Application to Use Vertebrate Animals in Research, Testing or Instruction

Project Title (If using external funds, enter the title used on the grant application): Investigating axonal retrograde transport of mitochondria in motor neurons of muscle-synthesized BDNF deficient mice

General Instructions
Please check the IACUC website to ensure you are using the current version of the form. All parts of this form must be submitted electronically to the Institutional Animal Care and Use Committee (email: IACUC@nmu.edu) and the relevant Department Head or other departmental designee. Review of this application will commence upon receiving the electronic application, but the project may not begin until all required approval signatures are obtained via Right Signature. 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 last Friday of every month. Applications for Full Committee Review must be electronically received by the first Friday of the month. Full Committee Review is required for applications that fall under USDA Use Categories D and E. Applications that fall under USDA Use Categories B and C will receive Full Committee Review 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): Erich N. Ottem, Ph.D.

Co-Investigator: Mikel Cawley

Department: Biology

Phone number: 906-227-1072

II. Funding Sources/Course Information and Dates
If the proposed work is for a course, please include the number of the course and title of the course

Funding Sources (External & Internal, if applicable) NIH/NINDS R15 AREA Grant Residual Funds Additional Funding Pending (click on the correct box)? ☐ Yes ☒ No

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Application Number: 311
Date Application Received: June 7, 2017
☒ Approved ☐ Denied on August 3, 2017

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Revised June 19, 2014 Check the IACUC website to ensure you are using the most recent form.
Project/Course Start Date: August 2017
End Date (three year maximum): August 2020

This application is (check one) ☒ New ☐ Modification of an application currently approved by the Institutional Animal Care and Use Committee (a new protocol must be submitted after three years)