Internal Faculty Grant Cover Sheet

Assessing Retrograde Transport Mechanisms in a Transgenic Mouse Model that Mimics the Pathology Associated with Amyotrophic Lateral Sclerosis

A proposal submitted to:
NMU Faculty Grants Committee

By

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Department of Biology
College of Arts and Sciences
Northern Michigan University

02 November 2015

Total Project Length:  (January 2016– July 2017)
Total Requested Funds: $ 6,877
Committed/Potential external funds: $
Total Project Budget:  $ 6,877

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I. SIGNIFICANCE

A. Statement of Need

The proposed research described in this grant application is a continuation of a long-term study that began nine years ago at Michigan State University during my postdoctoral fellowship and has been the main focus of my scholarly research at Northern Michigan University since arriving in 2008. Our laboratory focuses on the origins of pathology associated with neuromuscular diseases, in particular, the progressive neurodegenerative/myodegenerative disease amyotrophic lateral sclerosis (ALS; also known as Lou Gehrig’s Disease). In 2006, I developed a strain of transgenic mice that was missing a protein, brain-derived neurotrophic factor (BDNF), specifically in skeletal muscle. BDNF is normally synthesized by both spinal motorneurons and skeletal muscle fibers and acts in a kind of feedback system at synapses and the neuromuscular junction (NMJ) to maintain the integrity and strength of the connection between the pre- and postsynaptic structures. These mice proved to be very valuable to the ALS research community, and our laboratory was honored with a 3-year, $300,000 Academic Research Enhance Award (AREA; R15 grant) from the National Institutes of Health (NIH) in 2011. While these transgenic mice have provided great insight as to the consequences of losing BDNF synthesis from skeletal muscle as a result of the progressive pathology associated with ALS, our work continues. I have applied for a sabbatical for the 2016-2017 academic year to focus on advancing this research, training undergraduate and graduate students, and preparing a new AREA grant submission to the National Institute of Neurological Disorders and Stroke (NINDS) of the NIH. Happily, my sabbatical application was ranked first by the College of Arts and Sciences Advisory Committee, thus, I am very hopeful that it will be funded. For the sabbatical project to be successful, my laboratory needs a continuous source of funding, and during this “in
between grants period,” I heavily rely on the NMU Faculty Grants Program to allow my research efforts to continue uninterrupted. The preliminary data I was able to generate in 2009 and 2010 that led to the successful funding of our NIH grant was a direct result of a NMU Faculty Grant. 
To also note, these grants, both institutional and Federal, have allowed me to provide invaluable hands-on research experience to six graduate students and over 30 undergraduates in past several years. As you will note in my budget, all grant monies will go toward supplies to carry out the proposed research. Finally (and I have wrestled with whether to include this…deleting and “undoing” the deletion a number of times), these research efforts have become very personal to me. Seven years after I began this work, after the rather serendipitous development of the transgenic mice that now serve as unique and valuable models for ALS research, my father was diagnosed with the disease. He succumbed to the effects of ALS in July of 2013, six months after his diagnosis. This research grant application and all others to follow are dedicated to the memory of J. Norse Ottem.

**B. Literature Review**

This study aims to understand the role that the neurotrophic protein BDNF plays in the homeostatic maintenance of the neuromuscular junction (NMJ), the site of communication between motorneurons and the skeletal muscles they innervate. BDNF is a widespread protein, made by both neurons and by muscles, and is critical for maintaining the strength and integrity of synapses, dendrites, and other cellular structures (1-4). In general, the cellular roles and functions of BDNF that is synthesized by neurons are much better understood that its activity in other tissue types that synthesize it. For the last several years, our NIH-funded laboratory has attempted to address this gap in understanding, and in particular, delineate the importance of

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BDNF that is synthesized by skeletal muscle in the function of the entire motor unit—both the muscle fibers and the motor neurons that innervate them. For this, we have made use of a transgenic mouse line that is genetically engineered to have the BDNF gene deleted only in skeletal muscle. Thus, while neurons in the brain and spinal cord synthesize BDNF normally, the skeletal muscles of these transgenic animals do not. Using this model, we have come to understand the importance of muscle-synthesized BDNF and the implications for the onset of neuromuscular disease processes should this source of the neurotrophin become disrupted.

Our efforts, to date, have involved careful chronological assessment of pathology of skeletal muscles and motor neurons in mice missing a muscle-synthesized source of BDNF. At early ages (30 day old, 10 days post-weaning), mice display little neuropathology or myopathology; by 120 d (equivalent of older middle age), mice develop distinct myopathological markers including hypotrophic and hypertrophic myofibers, split skeletal muscles, and internalized myofiber nuclei (5). In addition, at 120 d, motor neurons begin to display progressive neuropathological markers. For instance, motor neuron cell bodies in transgenic mice at this age show signs of atrophy, and their dendrites have a significantly reduced diameter (5). Three other striking findings were noted from our pathological assessment of these mice. First, of the hindlimb muscles and associated motor neurons assessed for progressive pathology, the gastrocnemius (gastroc; commonly called the calf muscle)-associated motor units were much more susceptible to progressive pathology than any other neuromuscular systems. Second, while progressive myopathological and neuropathological processes did appear in later adulthood, transgenic mice did not exhibit an increased mortality rate, but did show several behavioral signs of an advancing neuromuscular disorder. Finally, in the course of assessing motor neuron pathology, we inadvertently uncovered evidence suggesting that disruption of a cellular process known as
retrograde axonal transport may underlie the progressive myopathy and neuropathology observed in transgenic mice missing a muscle-synthesized source of BDNF (5).

One does not often think of an enzyme or structural protein ‘getting old,’ but with constant use and age, proteins begin to lose their 3-dimensional structure. With this loss of shape, proteins also begin to lose their normal function. Retrograde axonal transport is a process by which older, misfolded, and dysfunctional proteins are packaged into vesicles in the distal synaptic terminals of neurons and then transported toward the cell body for recycling into component amino acids which can then be reused to make new proteins (6-9). If you consider a motorneuron that originates in your lumbar spinal cord and extends its axon to the extensor digitorum brevis muscle of your foot, one soon realizes the distances these proteins must travel to be recycled can be very great indeed! To maintain a healthy and functional NMJ, there is a constant stream of structural proteins and presynaptic-associated enzymes that are marked for recycling and subsequently retrogradely transported out of the motorneuron terminals. Any perturbation of retrograde axonal transport can lead to the accumulation of dysfunctional proteins in the NMJ, and this has been historically correlated with some of the neuropathologies and myopathologies associated with neuromuscular diseases such as ALS (10, 11).

We assessed retrograde axonal transport in our transgenic mice in two ways. First, we assayed the density of a structural protein, neurofilament-H (NF-H), in the distal motorneuron axons adjacent to NMJs of gastroc muscles in our transgenic mice. As strands of NF-H age, they become misfolded and have a phosphate group added by a kinase enzyme which marks them for retrograde transport and recycling (12). Thus, the retrogradely transported form of NF-H is referred to as phosphorylated neurofilament H (pNF-H). We observed that transgenic mice missing a muscle-synthesized source of BDNF had significantly more pNF-H accumulated in
distal motorneuron axons when compared to wild-type (WT), or control animals. These observations indicated that while phosphorylation of NF-H was occurring normally in transgenic animals, retrograde axonal transport mechanisms were being severely inhibited. A second means of assessing retrograde axonal transport is a sciatic nerve ligation assay. The sciatic nerve is actually a collection of motor axons that extends from the spinal cord and innervates several muscles in the hind limbs, including dense projections to the gastroc muscle. Physical ligation of the sciatic nerve leads to the accumulation of retrogradely transported proteins on the distal side of the ligation site and anterogradely transported proteins on the proximal side of the ligation site. We observed that transgenic mice had much less pNF-H accumulation at the distal ligation site when compared to WT animals. In addition, other commonly retrogradely transported proteins such as dynactin and synaptophysin did not accumulate at the distal ligation site. Ostensibly, these proteins, as was observed with pNF-H, were accumulating at the distal axons near the NMJ, unable to be moved retrogradely toward the cell body for recycling (13).

Progressive pathologies associated with the more common non-familial (non-genetic) form of the neuromuscular disease ALS, called sporadic ALS (sALS), have been compared to a wildfire (14): once started, the fire can move in almost any direction and spawn numerous secondary and tertiary fires. These multiple points of varied progressive pathology make the overall disease extremely difficult to treat. How can you address the mitochondrial dysfunction associated with ALS when reactive glia are compromising the motorneuron membrane? How can you inhibit reactive glia when retrograde axonal transport has stopped, and the motorneuron is disconnecting from the muscle at the NMJ (15)? It is possible that our transgenic mouse model has recapitulated at least one type of neuromuscular pathology that is among the multiple types of pathologies associated with the disease we refer to as ALS. Understanding how retrograde
axonal transport can be disrupted and how this disruption can ultimately be reversed will go far in slowing the entire disease process. However, further understanding of the nature of muscle-synthesized BDNF and its relationship to retrograde transport initiation in neuromuscular systems is critical to understanding the origin of NMJ pathology that is a component of the cellular degeneration associated with ALS progression.

C. Outcomes

The outcomes of this research (elaborated in the METHODS section) will provide new insight as to the role BDNF plays in the initiation and regulation of retrograde transport in neuromuscular systems. In addition, these efforts will begin to address the importance of maintaining a muscle-synthesized source of BDNF for the continued health of the entire motor unit. Finally, if our hypotheses are correct, these studies may demonstrate a specific mechanism that is disrupted following a loss of muscle-synthesized BDNF, and thus, provide further evidence that muscle neurotrophin replacement therapy may be a viable future treatment that may slow or alleviate progressive pathology associated with ALS.

II. METHODS

*Note: All procedures described below are currently approved by the Northern Michigan University Institutional Animal Care and Use Committee (IACUC), IAUCC Protocol 241 (Appendix C)*

a. **Determine the type, density, and phosphorylation state of the BDNF receptor at the NMJ.** The tyrosine receptor kinase B (TrkB) is the membrane-associated receptor through which BDNF binds and initiates intracellular signaling cascades. There are two isoforms of the
TrkB receptor. Full-length TrkB (fl-TrkB) is the most common isoform and mediates most BDNF signaling in the CNS. When fl-TrkB binds to BDNF, the receptor becomes internally phosphorylated which is a means by which active and inactive forms of the receptor can be distinguished (16). Truncated TrkB (tr-TrkB) is less common and little is known about its specific mechanism of action. To date, very little is known about the distribution or activity of TrkB receptors in neuromuscular systems.

Hypothesis: We predict that fl-TrkB expression will be present in the presynaptic membrane of motorneurons at NMJs of 120 d WT and transgenic mice. However, because transgenic mice are missing a muscle-synthesized source of BDNF, we predict that the ratio of phosphorylated (or active) fl-TrkB will be much higher in WT mice than in transgenic mice.

Methods and Analysis: We will use immunohistochemical assays and antibodies targeted against the non-phosphorylated and phosphorylated isoforms of the TrkB receptor as well as NMJ structural proteins. Multi-channel confocal microscopy will be used to visualize NMJs and TrkB isoforms. Images will be analyzed for TrkB expression density using Bitplane IMARIS ® image analysis software.

b. Determine the composition and density of retrograde transport complexes in the NMJ.

While largely uncharacterized in motorneurons, studies from pyramidal neurons of the hippocampus demonstrate that downstream signaling of BDNF via the TrkB receptor can initiate the formation of retrograde axonal transport complexes in the presynaptic bouton. Following BDNF binding, the TrkB receptor is phosphorylated and internalized by endocytosis from the presynaptic membrane. Following internalization, phospho-TrkB (p-TrkB) complexes with a group of proteins including dynactin and the motor protein dynein (17). Dynein is the protein...
that physically associates with the long, filamentous microtubules and “walks” the retrogradely transported cargo back toward the cell body. Dynactin acts as an adaptor to associate dynein with other proteins such as JNK-interacting protein 3 (JIP3) in the transport complex (18, 19). Studies of hippocampal neurons indicate that activated TrkB receptors are important for triggering transport complex formation (20).

Hypothesis: We predict that a significant portion of p-TrkB in the presynaptic motoneurons of the NMJ of WT animals will be internalized and in complexes with retrograde transport proteins such as dynactin, dynein, and JIP3. In contrast, we predict that we should observe little, if any, p-TrkB complexes with dynactin, dynein, and JIP3 proteins in motoneuron presynaptic NMJs of transgenic animals. Furthermore, we predict we will observe a lower density of retrograde transport complexes at the NMJs of transgenic mice.

Methods and Analysis: We will use immunohistochemical assays and antibodies targeted against p-TrkB, dynactin, dynein, JIP3 and NMJ structural proteins. Multi-channel confocal microscopy will be used to visualize NMJs and retrograde transport complexes. Images will be analyzed for p-TrkB presence in retrograde transport complexes using Bitplane IMARIS ® image analysis software.

c. Determine retrograde transport complex accumulation in a sciatic nerve ligation model.

To fully assess whether transgenic mice lacking muscle-synthesized BDNF also exhibit reduced TrkB-initiated retrograde axonal transport, we will determine the overall density of retrograde transport complexes (those positive for pTrkB and those negative for pTrK) at the distal site of a ligated sciatic nerve, as described in the background.
**Hypothesis:** We predict that transgenic mice will have much less overall accumulation of retrograde transport complexes accumulated at the distal side of a ligated sciatic nerve. Furthermore, we expect little, if any, pTrkB expression to be present in accumulated complexes.

**Methods and Analysis:** We will use immunohistochemical assays and antibodies targeted against p-TrkB, dynactin, dynein, JIP3 and axonal structural proteins. Multi-channel confocal microscopy will be used to visualize NMJs and retrograde transport complexes. Images will be analyzed for p-TrkB presence in retrograde transport complexes using Bitplane IMARIS ® image analysis software.

**D. Timeline**

- **January-April 2016:** Breeding of experimental mice
- **May- December 2016:** Training graduate and undergraduate students; sciatic nerve ligation protocols; Immunohistochemistry protocols
- **January-June 2017:** Image and data analysis; AREA (R15) preparation for June 2016 submission deadline.

**E. Qualifications**

I am well-suited to conduct all proposed experimentation and projects. I developed this animal model of neuromuscular disease in 2006 during my postdoctoral fellowship. I have worked extensively with these transgenic mice over the last 9 years using the experimental methods described in this application. I have been in the field of neuroscience and have been conducting physiological, behavioral, cellular, and molecular neuroscience research since 1998. My C.V. is included in Appendix B.

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The proposed budget items associated with my research proposal are chemicals, reagents, and antibodies needed for conducting the proposed assessment of neural tissue. No salary or fringe benefit-associated items are requested.

IV. PREVIOUS SUPPORT

My research has been funded by the Faculty Grants Program on two other occasions. I received a Faculty Grant in 2009 which led to the successful funding of an NIH AREA/R15 grant in 2011. In addition, in 2012, I received a NMU Faculty Grant to fund a collaborative project with my laboratory and bariatric physicians at Marquette General Hospital/Duke Lifepoint. I submitted a final project report summarizing the results of this latest project directly to the NMU Grants and Research Office on 31 October 2015 and have included a copy of the final report in Appendix D.
Appendix A.

REFERENCES


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CURRICULUM VITAE

CONTACT INFORMATION
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New Science Facility FAX: (906) 227-1063  
Northern Michigan University E-mail: eottem@nmu.edu  
Marquette, MI 49855

CURRENT POSITION  
Associate Professor, Biology Department,  
Northern Michigan University, Marquette, MI

EDUCATION
1998-2004  
University of Massachusetts-Amherst, Amherst, MA  
Ph.D., Neuroscience and Behavior Program, 2004

1992-1997  
Tennessee Technological University, Cookeville, TN  
B.S., Biology, 1997.

POSTDOCTORAL FELLOWSHIP
2004-2007  
Michigan State University, East Lansing, MI  
Postdoctoral Fellow, Breedlove-Jordan Laboratory

RESEARCH EXPERIENCE
Current Research  
Northern Michigan University, Marquette, MI  
Assistant Professor  
Focus: The role of neurotrophic factors in the prevention of  
neuromuscular disease

2004-2007  
Michigan State University, East Lansing, MI  
Post-doctoral Fellow Breedlove - Jordan Laboratory  
Focus: The influence of androgens and neurotrophins on the  
physiology of the motorneurons of the spinal nucleus of the  
bulbocavernousus.

1998-2004  
University of Massachusetts-Amherst, Amherst, MA  
Graduate Student, S.L. Petersen Laboratory  
Doctoral Thesis: Regulation of glutamatergic and GABAergic  
signaling by estrogen to promote gonadotropin hormone-  
releasing hormone surge release prior to ovulation in female rats
TEACHING EXPERIENCE

2008- Present  
**Northern Michigan University**, Instructor: (1) Human Anatomy and Physiology, (2) Neuroscience, (3) Intro to Cell and Molecular Biology, (4) Endocrinology, (5) Comparative Neuroanatomy  
**Responsibilities:** Design and deliver lectures, assignment, and assessments for pre-professional courses.

2007-2008  
**Michigan Technological University**, Instructor: (1) Neuroscience of Motor Control (2) Fundamentals of Kinesiology  
**Responsibilities:** Designed and delivered lectures, assignments, and assessments for pre-professional course.

Spring 2006, 2007  
**Lansing Community College**, Instructor: Human Physiology  
**Responsibilities:** Designed and delivered lectures, assignments, and assessments, and taught the associated laboratory for a sophomore level, pre-professional course.

Fall 2006  
**Michigan State University**, Instructor: Brain and Behavior  
**Responsibilities:** Textbook choice, design and delivery of lectures and assessments, for a sophomore level, interdisciplinary Psychology course

PUBLICATIONS


Ottem C.V.
BOOK CHAPTERS


PUBLISHED ABSTRACTS


Abrahamsson, K. E. and Ottem, E.N. (2011) The Role of Brain Derived Neurotrophic Factor in Amyotrophic Lateral Sclerosis 17th Annual SAEOPP McNair/SSS Scholars Research Conference


Ottem, E.N. and Petersen, S.L (2003) Terminals containing vesicular glutamate transporter 2 and vesicular GABA transporter make synaptic contacts with luteinizing hormone-releasing hormone neurons. Society for Neuroscience, New Orleans, LA.

Ottem, E.N., Godwin, J.G., and Petersen, S.L (2002) Coexpression of vesicular glutamate transporter 2 and glutamic acid decarboxylase mRNAs in specific nuclei of the preoptic area. Society for Neuroscience, Orlando, FL.

Ottem, E.N. and Petersen, S.L. (2000) NMDAR1 mRNA is preferentially expressed by a medial subpopulation of LHRH neurons in the preoptic area. Society for Neuroscience, New Orleans, LA.


GRANTS AND AWARDS

2011: NIH/NINDS R15 AREA Grant, Awarded: $368,200
2011: NMU Faculty Research Grant, Awarded: $ 6,713
2010: NIH R15 AREA Grant: Submitted
2009: Faculty Teaching Release Time Award (Winter 2010), Northern Michigan University
2007: Faculty Research Enhancement Award, Michigan Technological University: $24,000
2004-07: National Institute of Neurological Disorders and Stroke Postdoctoral Training Grant
2002-04: National Institute of Mental Health Predoctoral Training Grant, University of Massachusetts-Amherst
2004: Annual Conference on Steroid Hormones and Brain Function, Young Investigator Award, Finalist
2002: Recipient of the 6th Annual Vincent G. Dethier Award, Neuroscience and Behavior Program, University of Massachusetts-Amherst
2000: Gordon Research Conference on Excitatory Amino Acids, Travel Award

SEMINARS

2010: “The Role of Brain-Derived Neurotrophic Factor (BDNF) in the Maintenance of Motorneuron Function and in the Onset of Neuromuscular Disease.” Tri-Beta Honors Society, Northern Michigan University
2006: “Androgen regulation of motoneurons of the spinal nucleus of the bulbocavernosus via brain-derived neurotrophic factor and glutamate systems.” Weekly Brain and Behavior
2005: “Making the Most of the Annual Society for Neuroscience Meeting as a Graduate Student,” Weekly Graduate Student Forum, Michigan State University.

2003: “Effective Lecture Techniques for Teaching Assistants”, Proseminar Series for First-Year Graduate Students, University of Massachusetts.

2001: “Regulation of glutaminase gene expression in GABAergic neurons of the AVPV,” Center for Neuroendocrine Studies Seminar Series, University of Massachusetts.

PROFESSIONAL SOCIETIES

- Society for Neuroscience
- Endocrine Society
- Society for Behavioral Neuroendocrinology

COMMITTEES AND SERVICE

Current Chair, Institutional Animal Care and Use Committee, Northern Michigan University

Current Member, Faculty Grants Committee, Northern Michigan University

Current Member, Budget Committee

2011: Member, Biology Dept. Executive Committee, Northern Michigan University

2011: Chair, Biology Dept. Academic Programs Committee, Northern Michigan University

2010: Member, Faculty Senate, Northern Michigan University

2008-2009: Member, Department Faculty Search Committee, Northern Michigan University

2004-2007: Summer School in Behavioral Neuroendocrinology, organizing committee, Michigan State University


2004-2007: Society for Neuroscience Brain Awareness Week, organizing committee, Michigan State University
Research and Academic Referees

Erich N. Ottem

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1401 Presque Isle Avenue, Northern Michigan University, Marquette, MI 49855
Telephone: 906-227-2941
E-mail: aprus@nmu.edu
Application Number: 241
Date Application Received: 06/07/2014
Date of IACUC Review: 06/27/2014

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

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

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

I. Principal Investigator (Must be a faculty member or Department Head)
   Erich N. Ottem, Ph.D.

   Co- Investigator
   Rebecca Dangremond

   Department
   Biology

   Phone number
   906. 227. 1072

   Date
   02/28/2014

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

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

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

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

   Additional Funding Pending? ☐ Yes ☒ No
This application is (check one) ☐ New* ☒ Modification of an application currently approved by the Institutional Animal Care and Use Committee

Previous Approval Dates

*A new protocol must be submitted after three years

III. SPECIES, NUMBER OF ANIMALS, AND USE CATEGORY

In the table below, provide the Species to be used, the Number of each Species to be used, and indicate the USDA Use Category for the proposed procedures. A rationale must be provided below the table for using USDA Categories D and E procedures.

Species
Indicate the species, using common names only, of each animal to be studied. Use additional sheets if necessary. A rationale for choosing this species must be provided in Part V of this application.

Number of Animals
In the table below, indicate the maximum number of animals that will be used during the project period (up to 3 years) for each species.

USDA Use Category
For each species to be used, indicate the Use Category for the methods described in this proposal. A description of each USDA Category is given below. A rationale for Use Category D and E procedures must be provided.

**USDA CATEGORY B:** Animals that will be bred or purchased for breeding, but not used for experiments. This includes breeders, offspring that cannot be used because of improper genotype or gender and any other animals that will not participate in research studies.

**USDA CATEGORY C:** Animals used in research, experiments, or tests which involve no pain or distress or only momentary or slight pain or distress that WOULD NOT REQUIRE anesthetic, analgesic or tranquilizing agents (for example: s.c., i.m., i.p., or percutaneous i.v. injection, PIT tag insertion, a brief period of restraint, tissue harvesting after euthanasia).

**USDA CATEGORY D:** Animals used in research, experiments, or tests where appropriate anesthetic, analgesic, or tranquilizing agents are required to avoid pain or distress (e.g., major and minor surgery, tissue or organ collection prior to euthanasia, retro-orbital blood collection, prolonged restraint accompanied by tranquilizers or sedatives).

Animals used in research, experiments, or tests that may cause pain or distress, which cannot be treated with an anesthetic, analgesic or tranquilizer, but the agent or procedure producing the pain/distress is immediately discontinued or the animal is euthanized to prevent pain and/or suffering.

**USDA CATEGORY E:** Animals used in research, experiments, or tests involving pain or distress where the investigator is unable or unwilling to administer anesthetic, analgesic or tranquilizing agents (e.g., studies which allow endpoints that are painful or stressful, addictive drug withdrawals without treatment, pain research, noxious stimulation).
<table>
<thead>
<tr>
<th>Genus / Species + Common Name</th>
<th>Number of Animals</th>
<th>USDA Use Category</th>
</tr>
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<tbody>
<tr>
<td>Mouse (Mus musculus)</td>
<td>120</td>
<td>D</td>
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**Justification for USDA Category D and E procedures:**

All animals used for this study will be classified under USDA Pain Category D. As described below, transgenic mice will be used to investigate potential disruption of retrograde transport in motor neurons associated with the loss of a neurotrophic protein from skeletal muscle. There are two directions of transport in the axons of motor neurons, anterograde (from the cell body to the axon terminal) and retrograde (from the axon terminal to the cell body). By tying off, or ligating, the sciatic nerve, it is possible to analyze axonal transport in the transgenic mice. The mice must be alive for either direction of transportation to occur. In order to study the transportation of materials along the axon in these transgenic mice, the mice must be alive, but deeply anesthetized, when the sciatic nerve is ligated, and remain alive for eight hours post ligation. Animals will be given analgesics prior to surgery. Eight hours post ligation, mice will be euthanized by overdose with sodium pentobarbital via intraperitoneal (IP) injection.

Some animals (see below) will receive muscle injections of a diffusible probe used to label mitochondria. The goal is to label mitochondria at the axon terminals of motorneurons innervating the injected muscle. Animals must be alive in order for the probe to be sequestered by the mitochondria. Four days following injection of the probe, the injected muscle will be harvested. Because we will be labeling proteins at both the motorneuron and muscle portions of the neuromuscular junctions, and because muscle proteins degrade rapidly following death (Takeichi, et al., 1984. *Biochemical Medicine* 32: 341-8), it is necessary that the animals be alive, but deeply anesthetized, when the muscle is removed. After the muscle is removed, the animals will be euthanized immediately by overdose with sodium pentobarbital via IP injection, while remaining under anesthesia.

**IV. DESCRIPTION OF PROJECT**

(Use nontechnical language that a layperson can understand). Provide a summary description of this research, testing or instructional project, including:

A. The aims and objectives of the work.

While some neuromuscular diseases (NMDs), such as Spinal Muscular Atrophy (SMA), can be linked to specific genetic mutations, only around 20% of all cases of the NMD amyotrophic lateral sclerosis (ALS) can be linked to a mutation in a single gene, SOD1. Amyotrophic lateral sclerosis is therefore characterized more by symptoms and progression than by genetics. In humans, ALS is characterized by the degeneration of motor neurons associated with muscle fibers, resulting in an inability to command the associated muscles. Neurodegeneration usually begins in the limbs and ends in the abdomen and diaphragm, where degeneration of these neurons leads to death of the patient by slow asphyxiation. It would be inappropriate to use a mouse model of ALS which mimics the SOD1 mutation when such a small percentage of cases are due to the mutation.

The disruption of a class of diffusible proteins, the neurotrophic factors, is recognized as playing a role in the onset and progressive pathology of many NMDs, including motor neuron diseases (MNDs) such as the prevalent, but poorly understood non-genetic forms of ALS. Brain-derived neurotrophic factor (BDNF) is essential for normal functioning of neuromuscular systems and is present in both motor neurons and
muscles. BDNF acts as a bi-directional messenger to convey signals that the motor unit is working and should be maintained. To date, studies have focused on disruption of BDNF made in motor neurons as a factor contributing to MNDs. The contribution of BDNF made by muscles to the health of the motor unit is unknown. Transgenic BDNF null mutant mice die soon after birth, and heterozygous BDNF knockout mice suffer from a myriad of behavioral and cognitive deficits that are not easily traced to specific problems in neuromuscular systems. To determine the specific contributions that muscle-derived BDNF provides to the neuromuscular unit, we created transgenic mice that are missing the BDNF gene only in skeletal muscle fibers. Initial behavioral characterization of these mice indicates they suffer from an adult onset loss of normal neuromuscular functioning. In addition, mice with missing muscle-derived BDNF have muscle fiber loss and markers of myopathology in limb skeletal muscle. Taken together, initial assessment of our transgenic mice suggests that the resulting phenotype has characteristics similar to established models of motor neuron diseases (MNDs) and muscular dystrophies (MDs).

We have found that 30 d and 120 d old mice lacking muscle-synthesized BDNF appear to have shortened dendrites. This could be due to a disruption of the transportation of a fluorescent tracer used to assess dendritic length. The fluorescent tracer was injected into the gastrocnemius, and following uptake by the associated motor neuron, was transported from the axon terminal to cell body of the neuron, where it then diffused into the cell body and dendrites. This direction of transportation is known as retrograde transport. Disrupted retrograde transport is a characteristic of multiple NMDs, such as spinal bulbar muscular atrophy. Additionally, in Huntington’s disease, the mutation of the protein huntingtin alters the retrograde transport of BDNF and its receptor in dendrites of corticostriatal neurons. These neurons eventually degenerate. However, when BDNF is reintroduced, there is an increased survival in the neurons.

**Specific Aim 1: Assess transgenic animals for disrupted retrograde transport via sciatic nerve ligation.**

Our next study focuses on characterizing retrograde axonal transport in motor neurons in our transgenic mice. By tying off (or ligating) the sciatic nerve we will be able to investigate axonal transport in mice lacking muscle-synthesized BDNF. We plan on ligating the sciatic nerve at mid-thigh level in mice and, eight hours post-surgery, harvesting a segment of the sciatic nerve 5 mm both proximal and distal to the ligation site. The nerve segment will then be sectioned and immunohistochemistry (IHC) will be performed. The labels we intend to use are anti-BDNF, anti-synaptophysin and anti-phosphorylated neurofilament-H. Neurofilament H (NF-H) is a structural protein found in the axons of motor neurons. When NF-H is damaged, it is phosphorylated and recycled. A hallmark of ALS pathology is greater amounts of p-NF-H in motor neurons compared to control motor neurons. If there is a disruption of retrograde transport in mice with reduced or no muscle-synthesized BDNF, we expect to see an accumulation of p-NF-H in the nerve tissue proximal, but not distal to the ligation site. If retrograde transport is disrupted we also expect to see accumulation of the vesicle associated protein, synaptophysin, proximal but not distal to the ligation site.

**Specific Aim 2: Assess transgenic animals for mis-regulation of dynactin-1 in motorneurons.**

As mentioned above, signaling cascades activated by BDNF are important in maintaining a healthy motor unit. In the motorneuron soma, BDNF regulates the transcription of various genes. One gene in particular that is often misregulated in neuromuscular diseases is DCTN1, the gene coding for dynactin 1. Dynactin 1 is associated with the motor protein dynein, which transports materials in the retrograde direction. Dynactin 1 acts as an adaptor, linking the dynein motor to its cargo. We aim to investigate whether dynactin 1 levels in the cell soma of motorneurons are decreased in animals with reduced muscle-synthesized BDNF.

To do this, we will inject Fluorogold, a retrograde tracer used to label the somas and dendrites of motorneurons, into the gastrocnemius (GC) muscle. Following injection into the GC muscle, fluorogold is internalized by the motorneurons associated with the GC muscle, it is then retrogradely transported in vesicles to the soma, where it then diffuses into the soma and dendrites of the motorneuron. Muscles will be injected one week prior to spinal cord harvest. When the animals have reached the desired age (either 30 d or 120 d old), they will be euthanized, and spinal cords will be harvested and sectioned. IHC will be performed on spinal cord sections using anti-fluorogold and anti-dynactin 1 to label fluorogold and dynactin 1 respectively. We will quantify dynactin-1 that co-localizes with GC associated motorneurons (identified by fluorogold labeling) in both knockout groups and the control group. We hypothesize that animals with decreased muscle synthesized BDNF will have lower levels of dynactin 1 compared to control animals.
there are lower levels of dynactin 1 in animals with reduced muscle synthesized BDNF, this would suggest that BDNF is an important regulator of DCTN1 expression. It could also provide a possible mechanism for disrupted retrograde transport, if such is the case. With decreased dynactin 1, material cannot be efficiently transported retrogradely from the axon to the soma. This could lead to an accumulation of p-NF-H (described above), a decrease in cellular recycling, and a decrease in cell signaling, all of which would be damaging to the motorneuron, and as a result, the motor unit as a whole.

**Specific Aim 3: Assess transgenic mice for accumulation of mitochondria at the neuromuscular junction.**

Another characteristic of many neuromuscular diseases is oxidative stress caused by “leaky” mitochondria accumulated at the axon terminal. We aim to investigate if such accumulation is seen in the axon terminals of motorneurons in homozygous and heterozygous muscle-synthesized BDNF knockout mice. We will do this using MitoTracker™Red CMXRos, a diffusible, fixable, mitochondrion-selective probe that is used to label active mitochondria. We will inject MitoTracker™ into the GC of mice four days before they have reached the desired age (either 30 d or 120 d old). Once animals are of age (30 d or 120 d old), they will be highly anesthetized while the GC muscle is removed. Once the muscle has been harvested, animals will be euthanized. The muscle will be sectioned and subsequently labeled with anti-synaptophysin, α-bungarotoxin and anti-neurofilament H via IHC. Synaptophysin, a vesicle-associated protein, will be used to identify the pre-synapse at the neuromuscular junction, while α-bungarotoxin, an acetyl choline receptor marker, will identify the post-synapse. Neurofilament-H is a structural protein found in motorneuron axons. The three of these labels combined will represent the motor unit. MitoTracker™ that co-localizes with synaptophysin (because MitoTracker™ will label mitochondria in both motorneurons and muscle cells, and we only are concerned with mitochondria at the axon terminal of motorneurons) will be quantified.

We hypothesize that animals with reduced muscle-synthesized BDNF will have mitochondria accumulation at the axon terminal. This would further support potential disruption of retrograde transport. Also, as dynactin 1 is an adaptor protein that links dynein to its cargo, which includes autophagosomes containing mitochondria, a decrease in dynactin 1 could be responsible for the accumulation of mitochondria at the axon terminal. As mitochondria get old, they begin leaking reactive oxygen and nitrogen species (ROS and RNS respectively). These are cytotoxic to the cell. In a healthy motorneuron, old mitochondria get packaged up and transported retrogradely to the soma, where it is recycled. If retrograde transport is disrupted, old mitochondria may accumulate and leak ROS and RNS at the axon terminal. This is a possible mechanism for motorneuron degeneration in animals with reduced muscle-synthesized BDNF.

B. The significance of this work and expected outcomes.

If retrograde transport is disrupted in these mice, a possible avenue of treatment may involve the delivery of BDNF and other neurotrophins to muscles. This research project aims to determine the role that BDNF produced by muscles plays in maintaining axonal transport. Results may provide new insight into avenues of treatment for human neuromuscular diseases such as ALS.

V. JUDICIOUS USE OF ANIMALS

Guidelines issued by the United States Government entitled Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research and Training and University policy require that animals selected for a procedure should be of an appropriate species and that the minimum number required to obtain valid results should be used. Assistance is available at the University library and guidance may also be obtained from the IACUC web page or any IACUC member.

A. Provide a rationale for the selection of the animal species that will be used in this project. Describe the biological characteristics of the animal that are essential to the proposed study. Describe any experience with the proposed animal model and methodologies.

Our research involves investigating the role that muscle-synthesized BDNF plays in the maintenance of retrograde transport. Because BDNF is synthesized in the motor neurons and in the muscles and can act...
as both an anterograde and retrograde signaling molecule, the relative role played by the portion originating in the muscle is difficult to determine.

Using Cre/lox transgenic technology, we have the ability to target the BDNF gene for removal in muscles only. In this way, we can determine the role that muscle-derived BDNF plays in the maintenance of normal neuromuscular function by observing the behavior, physiology, and anatomy of animals that are missing a muscle source of the neurotrophic factor. Already, experimental mice have yielded data that suggest that absence of muscle-derived BDNF leads to neuromuscular deficits in adulthood, similar to mouse models of amyotrophic lateral sclerosis (ALS).

Experimental transgenic animals missing a muscle source of BDNF are not available commercially and can only be generated via a breeding program from a colony that houses two different strains of transgenic mice: Cre+ mice and BDNFlox+ mice. To maintain a supply of experimental animals, we must sustain these founding lines in a colonial breeding population.

The Principle Investigator (PI) of this proposal has over seven years of experience working with both the experimental strain of transgenic mice and the founding transgenic lines. Additionally, the PI has over fourteen years of experience working with rodent species involved in neurophysiological and neuromuscular research.

B. Provide a rationale for the number of animals listed in question #1. Describe the size and number of experimental groups and the number of animals needed for the procedures.

The number of animals in Category D (n=120) that will be used has been calculated to provide the minimal number of subjects to reliably analyze retrograde transport our transgenic mice. We strive to use the minimum number of animals that will allow us to reach conclusions on the basis of statistical significance.

We estimate a few animals that are present at the beginning of a study may fail to provide useful, analyzable tissue by the end of the study. For example, when performing nerve ligation to study axonal transport, if the nerve becomes nicked or is tied improperly, it will be impossible to gather the correct information from that particular animal.

For this proposal, we conducted a power analysis to determine the minimum number of animals to be used in this between-groups study. To reveal statistically significant effects using analysis of variance (ANOVA) statistical methods with a medium (0.30) effect size and suitable power (power=0.80, α =0.05), 8 subjects are necessary for each group in each study. There may be up to a 20% failure rate due to unexpected death of animals under anesthesia, tissue sectioning error, or similar, thus, we have added an additional 2 animals per group in each study. There will be three groups total: wild type controls, heterozygous knockouts and homozygous knockouts, and two age groups total: 30 d and 120 d old. A total of 120 mice are required for the proposed studies as outlined below:

| Experiment: Sciatic nerve ligation to address specific aim 1. |  |
|---|---|---|---|
| Number of Age Groups | Number of Experimental Groups / Age Group | Number of Animals / Experimental Group | Total Animals in Experiment |
| 2 | 3 | 10 | 60 |
Experiments: Intramuscular injections to address specific aims 2 and 3. Each animal will receive Fluorogold injections in the right leg (specific aim 2) and MitoTracker™ injections in the left leg (specific aim 3).

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<thead>
<tr>
<th>Number of Age Groups</th>
<th>Number of Experimental Groups / Age Group</th>
<th>Number of Animals/ Experimental Group</th>
<th>Total Animals in Experiment</th>
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<tr>
<td>2</td>
<td>3</td>
<td>10</td>
<td>60</td>
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C. The Federal Animal Welfare Act requires that you describe how you determined that acceptable alternatives (e.g., mathematical models, computer simulation or in vitro biological systems) to the use of animals in this project are not appropriate. Provide date(s), database(s), search terms used for a documented literature search indicating that alternatives do not exist. For teaching projects, the search should include literature to address educational issues as well.

Disruption of retrograde transport, mis-regulation of dynactin-1 expression and accumulation of mitochondria at the axon terminals of motorneurons have been observed in various neuromuscular diseases including Huntington’s disease, spinal bulbar muscular atrophy, and ALS. Our goal is to study the role muscle-synthesized BDNF plays in maintaining normal cellular transport. We want to investigate whether there is a disruption in retrograde transport when muscle-synthesized BDNF is decreased or knocked out. We then want to investigate a possible cause of disruption, and possible mechanisms for motorneuron degeneration as a result of disrupted retrograde transport. It is possible to culture motor neurons from embryonic stem cells or induced pluripotent stem cells. However, the lifespan of these motor neurons are 3-4 weeks. We have seen pathology that may be due to disrupted transport in skeletal BDNF KO or reduced 120 d old transgenic mice, and we not only want to further investigate transport in these mice, but also to develop a timeline to understand when this pathology begins to appear. This isn’t possible using cultured motor neurons with such a short lifespan. Also, because we are studying the role of muscle-synthesized BDNF in maintaining the health of neuromuscular units, using cultured motor neurons is not appropriate. Even if motor neurons can form neuromuscular junctions when co-cultured with myocytes, exercise is required for the release of BDNF from muscle. For these reasons, it would be inappropriate to use cell culture to study retrograde transport in vitro. A computer approach would also have limited use in such a study.

Our first step in investigating the possible disruption of muscle synthesized BDNF is to perform sciatic nerve ligation. This procedure isn’t something we could do to individual neurons in vitro. Not only is the sciatic nerve conveniently large, but its ligation allows us to investigate the activity of multiple neurons at once. Because we are asking questions regarding neuromuscular systems that are influenced by many factors, not all of which are known, a transgenic mouse model is the only way to sufficiently find the answers.

(Date of search: 4/9/2014; Database: PubMed; Keywords: motor neurons, computer model, motor neuron disease, atrophy, neurotrophic factors, BDNF, immortalized cell line, in vitro, neuromuscular development).

VI. SOURCES, HUSBANDRY, AND LOCATION OF ANIMALS

A. How will animals be acquired? Include specific vendor, where applicable. If animals are being drawn from a currently approved NMU animal colony, please indicate this here and include the IACUC colony approval number.
Experimental animals will be obtained via a breeding program using transgenic breeders from a colony maintained in the Northern Michigan University Biology Department.

B. Housing conditions Describe (briefly using nontechnical language) the care and conditions that will be provided for the animals if they are to be held prior to or following the experimental procedure. Include proposed recordkeeping protocols and duration of the non-experimental housing period.

I. HOUSING CONDITIONS AND ANIMAL HUSBANDRY

A. Housing

Experimental mice will be housed in 19.56cm x 30.92cm x 14.93cm cages, no more than 4 mice per cage.

B. Husbandry

1. Daily Health Observations

Animals will be observed for health concerns 365 days a year including holidays and weekends.

   i. If health problems are observed the following steps will be taken:

      a) Record abnormal observations (not diagnoses) on Animal Health Form that is associated with each colony.

         -Record illness, injury, deaths, or births, flooded cage, fighting, etc.
         -Signs of illness: Ocular or nasal discharge, pale, excessive salivation, sneezing, rough hair coat, hair loss, anorexia, weight loss, adipsia (not drinking), decreased or no feces, decreased or anuria (not urinating), polyuria (copious urine; bedding may contain more urine than other cages), diarrhea, depression, ataxia (movement problems) change in skin color, masses/ lumpy, skin ulcerations, enlarged abdomen, continuous scratching, etc.

      b) Report health problems to animal facilities veterinarian

      c) Employ treatment or procedure recommended by veterinarian

   ii. If dead or moribund animals are discovered:

      a) Report immediately to animal facilities veterinarian for consultation regarding animal sequestering

      b) Following consult, moribund animals will be euthanized, and carcasses of animals will be placed in a ziplock bag and placed in a freezer prior to pick-up for disposal.

2. Daily Husbandry - 365 days a year (includes Holidays and weekends)

   I. Food

      a) Animal will be fed ad lib; Animals should always have access to food.

      b) Palatability of food is will be ensured daily - i.e., food not expired, not contaminated with debris, water, mold, or otherwise spoiled or rancid. contaminated food will be removed.
II. Water
   a) An adequate water supply will be ensured daily - Animals should always have access to water.
   b) If water is cloudy or has debris - empty bottle, rinse well and refill / or change bottle.
   c) Water consumption will be monitored to ensure animals are drinking.

III. Update cage cards
   a) Animal identification updates
   b) Update procedure cards

IV. Room Conditions
   a) Room temperature will be recorded (acceptable temperature: 72 °F +/- 4°C), and room humidity will be recorded (acceptable humidity: 55% +/- 15%). See attached Daily Room Conditions Form.
   b) Experimental animal census will be recorded. See attached Experimental Animal Census Form.
   c) Any room problems will be noted (i.e., vermin, escaped animals, spills, leaks, lights, electrical, plumbing, cooling or heating malfunctions):
      i. Room problems will be documented.
      ii. Department Head will be immediately notified.

3. 1-3X / Week Duties and Husbandry

I. Change rodent cages 1X/per 9 days
   a) No more than 1 inch of new bedding will be placed in the new cage.
   b) Animals are transferred to the clean cage.
   c) Food and water bottle is transferred to the clean cage. More food and water will be added if needed.
   d) Cage and procedure cards will be transferred to the clean cage.

II. Water bottles and stoppers will be changed weekly.

II. EXPERIMENTAL ANIMAL RECORDS

1. Experimental Animal Identity Records
   Records for experimental animals will be kept in electronic and cage card form. Both electronic and cage card records will contain the following information:
   PI Name and IACUC number
   Designation as Experimental Animal
   Experimental genotype designation
   Cage number
   Animal ID number
   Parental ID
   Sex
   PCR date (to establish genotype)
   Date of Birth
   Ear Punch Identification key
   Genotype
35

Erich Ottem  IACUC#XXXXXXXX
Experimental Animals
Experimental HSA-Cre BDNFlox

Animal Supplier: IACUC #

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<tr>
<th>Animal ID</th>
<th>Parent ID</th>
<th>Sex</th>
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<th>DOB</th>
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2. Procedure Records

Additional electronic and cage card records for each animal describing any procedures undergone, such as intramuscular injection. In addition, euthanasia records for individual animals will be kept as part of a procedural record.

Example

**Procedure Card**

Animal ID: 24 BDNF
DOB: 10/30/2008
SEX: F

<table>
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<tr>
<th>DATE</th>
<th>PROCEDURE</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>i.m. injection ID:</td>
</tr>
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</table>

C. Will the animals undergo multiple survival surgical procedures (counting surgical procedures from previous protocols)?

☐ Yes  ☒ No

D. If yes, please provide justification.

E. Will any animals from this protocol be used in other projects at a later date?

☐ Yes  ☒ No

If yes, please be aware that IACUC approval is required prior to transfer of the animal

F. Will this project use dogs, cats, nonhuman primates or farm animals?

☐ Yes  ☒ No

If yes, describe the quarantine and conditioning procedures to be performed prior to the start of the project. Include selection criteria that will be used in including or excluding an animal from this project.

G. Location of animal housing:

Building

Biology Animal Facility
VII. EXPERIMENTAL PROTOCOL

For each species, describe the procedures to be employed (e.g., surgery, behavioral training, administration of substances for testing, etc.). Be certain to include information under the following headings:

A. Procedure Details of the procedure to be performed on each animal including the time frames and intervals in the order in which each procedure will be performed. Indicate if the procedures are to be performed on the animals or tissues.

For Specific Aim 1

Animals will be administered the analgesics Buprenorphine and Meloxicam prior to surgery. Animals will be deeply anesthetized and an incision will be made in the right lower limb. The right sciatic nerve will be exposed and a 7-0 silk suture will be tied tightly around the nerve at mid-thigh. The incision will be closed using 7 mm stainless steel wound clips (Harvard Apparatus). After eight hours, animals will be euthanized by overdose with sodium pentobarbital (via IP injection) and perfused with 4% paraformaldehyde fixative in phosphate buffer, pH 7.4. A segment of the right sciatic nerve, including 5 mm both proximal and distal to the ligation site, will be removed. The non-ligated, left sciatic nerve will also be removed in the same manner as the right nerve.

To begin, in order to fine tune medication doses, surgery will be performed on one practice animal. The animal will be closely monitored for pain throughout the entirety of the procedure, from the first analgesic administration until the sodium pentobarbital overdose. Analgesic doses will be adjusted based on pain or sedation levels.

All animals will be placed in a clean cage and kept separate post-surgery. All animals will be monitored every 30 min post-surgery for overt signs of pain including vocalizations, repetitive behaviors, excessive cowering, shaking etc. Respiratory rate will be monitored visually. If mice do begin to show signs of undue pain, a follow up dose of buprenorphine will be administered.

For Specific Aims 2 and 3

For the following procedures, all equipment and tracers will be sterile.
Animals will be administered Buprenorphine prior to muscle injections. Seven days prior to the desired end point (30 d or 120 d old), Fluorogold will be injected into the right GC muscle. Fluorogold is a retrograde tracer used to label the soma and dendrites of motorneurons. Animals will be deeply anesthetized using isofluorane, and the GC muscle will be exposed by a single incision of the skin 5-15 mm long. Each animal will receive three injections into the right GC muscle, each injection supplying 3 µl of Fluorogold. This procedure will not require opening a body cavity, and is therefore not considered a surgery. Incisions will be closed with stainless steel 7mm wound clips (Cell Point Scientific). Wounds will be swabbed with betadine and each animal will be placed in a clean cage to recover. Mice are typically ambulatory within an hour. Three days later, animals will receive 3 injections, each supplying 2 µl of MitoTracker™, into the left GC muscle following the same protocol. MitoTracker™ is a diffusible probe used to label active mitochondria.

When animals have reached the desired age (30 d or 120 d old), animals will be deeply anesthetized using isofluorane and the left GC muscle (the MitoTracker™ injected muscle) will be removed. Immediately following removal of the left GC muscle, animals will be euthanized by sodium pentobarbital overdose via IP injection, and intracardially perfused with saline and fixative (i.e., 4% paraformaldehyde in phosphate buffer). The spinal cord of each animal will then be removed to assess dynactin-1 levels in the somas of motorneurons.

B. Pharmacological Agents
Describe the use of test substance(s), or agent(s). Include a description of the dose(s) and route(s) of administration (if applicable).

- 9 ul 3% Fluorogold, 1% DMSO in saline, intramuscular injection

- 6 ul 150 nM MitoTracker™ Red CMX Ros. Stock solution will be prepared by dissolving MitoTracker in DMSO to a final concentration of 1 mM. This stock solution will then be diluted with 0.9% saline to the final working concentration of 150 nM. Route: Intramuscular injection

Describe the use of anesthetic, analgesic, and tranquilizer agent(s). Include a description of the dose(s) and route(s) of administration. Indicate how the animals will be monitored during anesthesia and during post-anesthesia recovery (if applicable)

Analgesic
- Buprenorphine (1.5 mg/kg SC q6-12 hr); administered 20 minutes prior to surgery

Anesthetic
- Isoflurane (0.1-0.5% via nose-cone with monitoring; inhalant); used for deep anesthesia during surgery

Non-Steroidal Anti-Inflammatory drug
- Meloxicam 2.5 mg/kg PO q24 h; administered 2 hr prior to surgery

Analgesic doses may be adjusted based on pain levels and or sedation noted at the starting dose.

C. Euthanasia Describe the methods of euthanasia that will be employed including how you will ensure that the animal is deceased.

Animals will be euthanized by overdose with pentobarbital. Tail and foot pinches will be done to ensure that the animals are deceased. Following sodium pentobarbital overdose (via IP injection), mice will be perfused with 4% paraformaldehyde fixative in phosphate buffer, pH 7.4.
D. **Adverse Effects**  Describe (using nontechnical language) any anticipated adverse effects on the animals well-being.

Possible adverse effects include accidental anesthetic death, unexpected levels of pain, motor disabilities due to nerve ligation, as well as surgery complications such as nicking the femoral artery or vein. Animals will be monitored closely. If inadvertent serious injury to an animal does occur, the animal will be euthanized immediately by rapid CO₂ asphyxiation.

F. **State and Federal Assurances for Field Studies (if applicable):** If the proposed studies will be conducted in the field, then describe any state and federal assurances (e.g., a permit) that you have obtained or will obtain before conducting these studies.

G. **Provide additional comments as necessary.**

---

**VIII. PERSONNEL INVOLVED IN PROJECT**

List dates and types of animal-related training completed by personnel involved in the project and check off activities that will be performed by personnel. Please refer to current training requirements as listed on the IACUC web page. Should personnel change during the project, please inform IACUC prior to personnel undertaking any project-related activity via written memorandum. In this memo, supply similar information about their training for their project-related activities. If you have further questions, please contact the chair of the IACUC or the Institutional Officer.

<table>
<thead>
<tr>
<th>Personnel Name</th>
<th># Years experience with specified animals</th>
<th>Will provide animal husbandry</th>
<th>Will conduct non-surgical experimental procedures</th>
<th>Will conduct surgery or anesthesia (specify)</th>
<th>Will/may perform euthanasia</th>
<th>CITI Program Module(s) Completed</th>
<th>Date Module Completed</th>
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<tr>
<td>Erich N Ottem Ph.D.</td>
<td>15</td>
<td>Yes</td>
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<td>Yes (both)</td>
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<td>4/1/2012 4/1/2012 4/1/2012 4/1/2012</td>
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<td>Rebecca Dangremond</td>
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<td>Amanda Taisto</td>
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<td>Ryan Brandt</td>
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<td>Stephanie Kropf</td>
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<td>Jenna E</td>
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14
IX. OCCUPATIONAL SAFETY AND HAZARDOUS MATERIALS

A. Does the research, testing, or instruction require the use of hazardous agents (i.e., infectious agents, carcinogens, toxic chemicals, radioisotopes)?
   - Yes  - No

B. If Yes, specify the hazardous agents to be used and describe their hazards. For those agents classified as NEPA hazards greater than Level 1 or that are unclassified electronically attach the appropriate MSDS with this application when you submit it by email.

C. Is the hazardous agent a biohazard?
   - Yes  - No

D. If yes, specify Biohazard Safety Level

E. Describe the containment protocol to be followed in protecting other animals and personnel from hazardous agents, and if applicable, isotope or controlled substance license/permit. If the containment protocol used is one recommended by a governing body, i.e. Nuclear Regulatory Commission, in addition to describing the protocol you will use for containment, provide a copy of the organization's guidelines with relevant sections highlighted or tabbed. Do not merely state "hazard will be contained based on protocol suggested by U.S. Centers for Disease Control."
I acknowledge responsibility for this project. I have read the Northern Michigan University Principles for the Care and Use of Laboratory Animals and certify that this project will be conducted in compliance with those principles. I assure that I will obtain Institutional Animal Care and Use Committee approval prior to significant changes in the protocol. I assure that this project does not unnecessarily duplicate previous research or instructional projects. I assure that students, staff and faculty on the project are qualified or will be trained to conduct the project in a humane, safe, and scientific manner.

Signature: ____________________________________________________
Principal Investigator Date

XI. APPROVAL OF SCIENTIFIC MERIT (to be completed by the Department Head)
Before it is initiated, this project must be reviewed and approved on the basis of its scientific merit.

☐ Review conducted by external agency.
☐ Governmental Agency: Please specify the reviewing agency or board Federal agency (e.g., NIH, NSF, USDA, etc.) and evidence of approval

☐ Nongovernmental agency (e.g., University review, Other specify):

☐ Departmental Review: I assure that this project has been reviewed and approved for scientific or instructional merit by:

☐ Expert reviewer (Name)

☐ Departmental Committee Review (Committee Name and Chairperson):

☐ Other (Describe):

Signature: ____________________________________________________
Department Head Date

XII. REVIEWED AND APPROVED BY IACUC REVIEWERS

Signature: ____________________________________________________
Institutional Animal Care and Use Committee Chair Date

Signature: ____________________________________________________
Institutional Animal Care and Use Officer Date

Following action on this application, copies of approval or denial letters will be sent to the applicant, Department Head, and appropriate College Dean who will also receive a copy of this application.
SIGNATURE PAGE

IACUC #: 241  PROPOSAL TITLE (From cover page): Investigating axonal retrograde transport in motor neurons of muscle-synthesized BDNF deficient mice

X.  ACKNOWLEDGEMENT BY PRINCIPLE INVESTIGATOR

I acknowledge responsibility for this project. I have read the Northern Michigan University Principles for the Care and Use of Laboratory Animals and certify that this project will be conducted in compliance with those principles. I assure that I will obtain Institutional Animal Care and Use Committee approval prior to significant changes in the protocol. I assure that this project does not unnecessarily duplicate previous research or instructional projects. I assure that students, staff and faculty on the project are qualified or will be trained to conduct the project in a humane, safe, and scientific manner.

Signature: ___________________________  06/06/2014
Principal Investigator

XI.  APPROVAL OF SCIENTIFIC MERIT (to be completed by the Department Head)

Before it is initiated, this project must be reviewed and approved on the basis of its scientific merit.

☐ Review conducted by external agency.

☐ Governmental Agency: Please specify the reviewing agency or board Federal agency (e.g., NIH, NSF, USDA, etc.) and evidence of approval

☐ Nongovernmental agency (e.g., University review, Other specify):

☐ Departmental Review: I assure that this project has been reviewed and approved for scientific or instructional merit by:

☐ Expert reviewer (Name)

☐ Departmental Committee Review (Committee Name and Chairperson):

☐ Reviewed by Department Head

☒ Other (Describe):

Signature: ___________________________  06/06/2014
Department Head

XII.  REVIEWED AND APPROVED BY IACUC REVIEWERS

Signature: ___________________________  06/06/2014
Institutional Animal Care and Use Committee Chair
Following action on this application, copies of approval or denial letters will be sent to the applicant, Department Head, and appropriate College Dean who will also receive a copy of this application.
Signature Certificate

Document Reference: IBLS6RIXCLVH9PSYIR7FNR

Brian Cherry
Party ID: K4F4PU4B43LV724P7YG73N
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Multifactor Digital Fingerprint Checksum: cd1ca45d7c1a51932540865736103ce3b30d33d

John Bruggink
Party ID: LHW7K5IJ3LGH95G2ASTWSB
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VERIFIED EMAIL: jbruggink@nmu.edu

Multifactor Digital Fingerprint Checksum: 12a74dfb0c12a507915e008b15c595e056766247

John Rebers
Party ID: M54LWMISJ4V4JE9CAV2MD
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Multifactor Digital Fingerprint Checksum: 4d43a8f4885d4257c1a5f8a1e4cc37ca8e4c0494

Erich Ottem
Party ID: E259GPIJWJ7SLAAPFC5JI
IP Address: 35.24.121.74
VERIFIED EMAIL: eottem@nmu.edu

Multifactor Digital Fingerprint Checksum: 9016e63a29752237df90d048f489f4250e2607

This signature page provides a record of the online activity executing this contract.
## Audit

All parties have signed document. Signed copies sent to: Michael Broadway, LuAnne Crupi, Brian Cherry, John Bruggink, John Rebers, and Erich Ottem.

Document signed by Brian Cherry (bcherry@nmu.edu) with drawn signature. - 97.95.80.61

Document viewed by Brian Cherry (bcherry@nmu.edu). - 97.95.80.61

Document signed by John Bruggink (jbruggin@nmu.edu) with drawn signature. - 35.24.3.145

Document viewed by John Bruggink (jbruggin@nmu.edu). - 35.24.3.145

Document signed by John Rebers (jrebers@nmu.edu) with drawn signature. - 35.24.3.43

Document viewed by John Rebers (jrebers@nmu.edu). - 35.24.3.43

Document signed by Erich Ottem (eottem@nmu.edu) with drawn signature. - 35.24.121.74

Document viewed by Erich Ottem (eottem@nmu.edu). - 35.24.121.74

Document created by LuAnne Crupi (lcrupi@nmu.edu). - 198.110.203.50

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This signature page provides a record of the online activity executing this contract.
Hello LuAnne Crupi,

This document has been signed by Brian Cherry, John Bruggink, John Rebers, and Erich Ottem and is now complete.

Filename: IACUC_241_Ottem.docx
Reference #: IBLS6R italiane H9PSYIR7FNR
Subject: IACUC 241 - Signatures needed
Message: Please sign the final version of IACUC 241 as approved by the IACUC. When all required signatures are received, you will receive an e-mail with the final document attached.

**PIs - BE SURE TO ADD THE FINAL APPROVED PROTOCOL (WITH ALL SIGNATURES) TO YOUR IACUC DOCUMENTATION.**

NOTE-Deans are now being copied on this process so that they can be aware of approved protocols.

Contact iacuc@nmu.edu if you have any questions.

For your convenience, a pdf of the completed document is attached to this email.

To view this document on RightSignature, follow this link:
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Erich N. Ottem, Department of Biology
NMU Faculty Grant, 2012-14

Project: Investigating the Influence of Exercise on the Neuroendocrine Regulation of Feeding Behavior in Mice on a High-Fat Diet

What Was Done? (Summarize the project activities.)

With the funding provided by the NMU Faculty Grant we advanced the project in a number of areas:

Animal Experimentation

- We purchased animal behavioral testing equipment which included running wheels with digital counters to record the voluntary running behavior of experimental mice.

- We purchased specialized foods used for high-fat diet experimentation so as to be able to precisely and accurately monitor the daily caloric consumption of our control and experimental mice.

Tissue Fixation and Histology Supplies

- We purchased a number of reagents required for tissue fixation for control and experimental neural tissue.

Immunocytochemistry Reagents

- We purchased a number of primary and secondary antibodies required for the visualization of neuroendocrine-associated neurons involved in the regulation of feeding behavior --- ghrelin and nesfatin-1 neurons in the hypothalamus. Additionally, we purchased primary and secondary antibodies to visualize phosphorylated mTOR, a marker of neural activation to determine which neuroendocrine system may be activated in mice allowed to voluntarily exercise and provided a high-fat diet. Finally, we purchased primary and secondary antibodies to label tyrosine hydroxylase so as to visualize dopamine neurons associated with the so-called “dopamine reward pathway.” Activation of dopamine neurons was determined by labeling the protein cFos using purchased antibodies.

Summary:

- Our purchases allowed us to assemble control and experimental groups as follows: (1) Control mice (a-group) : mice fed a normal diet and provided an immobilized running wheel; experimental mice (a-group): mice fed a normal diet and provided an active running wheel; experimental mice (b-group): mice fed a high fat diet and provided an immobilized running wheel; experimental mice (c-group): mice fed a high-fat diet and provided an active running wheel.

- The daily weight, caloric intake, and wheel revolutions were monitored for all mice for a six-week period.

- At the end of six weeks, mice were euthanized and tissues were collected to assay the activity of the hypothalamic...
neuroendocrine system involved in the regulation of feeding behavior. Additionally, we assayed the activity of dopamine reward pathway.

**What were the results?**

**Animal Behavior Studies**

One of the most striking results of our behavioral studies is depicted in the Figure 1. Control animals that were provided a standard diet and were sedentary (no access to a function running wheel; CS) gained significantly more weight that did animals on a normal diet that were provided a functioning running wheel allowing for voluntary exercise (CE). Mice on a high-fat diet that were also sedentary (HFS) gained significantly more weight than all groups. Interestingly, mice of a high-fat diet that were allowed voluntary exercise (HFE) gained significantly less weight that did HFS mice and were no different that CS mice. These results are significant for two reasons. First, our data clearly indicate that weight gain associated with a high-fat diet can be mitigated by voluntary exercise. Second, CS mice are essentially the standard mouse model used in countless biomedical studies and cross a multitude of disciplines. Given that CE mice gained significantly less weight that did CE mice, these results may indicate that researchers are not using the potentially healthiest mice as standard controls.

![Figure 1](image.png)

**Figure 1.** Average weight gain over 6-week period in diet and exercise groups. CE mice gained significantly less weight than CS mice (*, P<0.05). HFS mice gained significantly more weight than any other group (***, P<0.001). Additionally, HFS mice gained significantly less weight than did HFS mice (###, P<0.001) but were no different that CS mice.

As shown in Figure 2, CE mice, HFS mice, and HFE mice all consumed more calories in the six-week experiment than did the CS mice indicating that weight gain or loss was not solely correlated with the average calories consumed.
Neuroendocrine Studies

All immunocytochemistry assays were completed on control and experimental groups, and image analysis protocols were performed to determine whether there is differential activation of ghrelin or nesfatin-1 neurons in the hypothalamus in mice on a high fat diet that were sedentary or were provide voluntary exercise. Figure 3 depicts neurons in the paraventricular nucleus of the hypothalamus of a HFE mouse that are co-labeled for ghrelin, nesfatin-1, and phosphor-mTOR.

Figure 2. Average caloric intake over 6-week period in diet and exercise groups. CS mice consumed significantly less calories than did the CE, HFS, or HFE groups (*, P < 0.04)
Image analysis of tissue indicates that phosphor-mTOR is upregulated in nesfatin-1-positive neurons in both CE and HFE mice (Figure 4). These results indicate that exercise may increase nesfatin-1 neural activity to limit weight gain in HFE mice, and possibly lead to weight loss in CE mice (see Figure 1).

**Figure 3.** Confocal image of immuno-labeled hypothalamus neurons from a HSE mouse that express nesfatin-1 (red; Panel A), ghrelin (green; Panel B), and phosphor-mTOR (purple; Panel C). DAPI was used to label neural nuclei (blue; Panel E). Composite image is depicted in Panel E.
Dopamine Reward Pathway Studies

All immunocytochemistry assays were completed on control and experimental groups, and image analysis protocols were performed to determine whether there is differential activation of dopamine neurons in the ventral tegmental area (VTA) as denoted by expression of the immediate early gene, cFos. Figure 4 depicts dopamine neurons in the VTA of a HFS mouse that are co-labeled with cFos.

Figure 3. Results of image analysis measuring mTOR expression density in nesfatin-1 neurons in control and experimental groups. mTOR expression significantly increases in both CE and HFE groups. (**) P<0.001
Image analysis was performed to determine whether VTA dopamine neurons are activated in mice on a high-fat diet that are also provided with voluntary exercise. Similar to phosphor-mTOR expression patterns, c-Fos immunolabeling density was significantly increased in VTA dopamine neurons of both CE and HFE mice (Figure 5).

Figure 4. Confocal image of immune-labeled dopamine neurons (green; Panel A) also positive for the immediate early gene cFos (red; Panel B). DAPI was used to label neural nuclei (blue; Panel C). Composite image is depicted in Panel E.
Figure 5. Results of image analysis measuring cFos expression density in dopamine neurons of the VTA in control and experimental groups. cFos expression significantly increases in both CE and HFE groups. (**P<0.001)

What was produced (publication, presentation, creative work, etc.)?

This work is being prepared for submission to the peer-reviewed journal, *Hormones and Behavior*

Budget Expenditures

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