MOLECULAR MECHANISMS OF RADIAL AXONAL GROWTH: INSIGHTS
FROM ANALYSIS OF NEUROFILAMENT GENE-TARGETED MICE

A Dissertation
Presented to
The Faculty of the Graduate School
At the University of Missouri

In Partial Fulfillment
Of the Requirements for the Degree
Doctor of Philosophy

By
DEVIN M. BARRY
Michael L. Garcia, Dissertation Advisor

MAY 2012
The undersigned, appointed by the dean of the Graduate School, have examined the dissertation entitled

MOLECULAR MECHANISMS OF RADIAL AXONAL GROWTH: INSIGHTS FROM ANALYSIS OF NEUROFILAMENT GENE-TARGETED MICE

Presented by Devin M. Barry
A candidate for the degree of
Doctor of Philosophy of Biological Sciences
And hereby certify that, in their opinion, it is worthy of acceptance.

________________________________________
Dr. Michael L. Garcia, Dissertation Advisor

________________________________________
Dr. David Schulz

________________________________________
Dr. Samuel Waters

________________________________________
Dr. Douglas Anthony

________________________________________
Dr. Mark Hannink
ACKNOWLEDGEMENTS

I would first like to thank my mentor Dr. Michael Garcia. He has gone so far to impart his knowledge of biology to me, and I owe my development as a researcher to him. Dr. Garcia was understanding, patient, and thorough and always had an open door. The gratitude I have for all of his support through the years cannot be expressed, but I say thank you so much.

I would like to thank my committee members Dr. David Schulz, Dr. Samuel Waters, Dr. Douglas Anthony and Dr. Mark Hannink for their guidance and support. Dr. Schulz has been a great source of insight and knowledge into evolutionary biology and electrophysiology that helped with my research progress. Dr. Waters was a valuable source in my screening of ES cells for the generation of gene replacement mice. Dr. Anthony was a great source for better understanding neurofilament biology. Dr. Hannink gave me biochemical perspectives that greatly improved my understanding of my research.

I would next like to thank my wife and family for all of their support and believing in me. To my wife Rachel, your love and support have been unconditional and I could not have done any of this without you. To my newborn son Gavin, you have given me motivation and strength to be all that I can. To Mom and Dad, I could not have gotten to this point without all of your love and guidance through all the years. Kyle and Garet, you have not only assisted me
with my research in the lab but you have been the best brothers anyone could have. It was great to be so close with you through your college years.

I would like to thank the following former and current lab members: Hailian Shen, Cory Carpenter, Craig Yager, Ben Golik, Ginny Garcia, Natalie Downer, Jeff Dale, Stephen Shannon, Patricia Enmore, Eric Villalon, Nathan Byers, and Jon Strope. You made the lab a fun and enjoyable environment. I will miss going to CJ's for wings and beer with everyone!. All of you helped me in some way with my research and for that I am grateful.

Also thanks to our collaborator Nigel and his group for preparing many of my nerve root samples, performing nerve conduction velocity measurements on my mice and for insights that improved my research manuscript. Your work was much appreciated. I would like to thank Cheryl Jensen for all of her technical help with electron microscopy preparations and imaging. And thank you to everyone else that I did not mention but who helped me along the way.
TABLE OF CONTENTS

ACKNOWLEDGEMENTS........................................................................................................ ii

TABLE OF CONTENTS........................................................................................................ iv

LIST OF TABLES................................................................................................................ vi

LIST OF FIGURES............................................................................................................... vii

ABSTRACT............................................................................................................................ ix

CHAPTER 1 INTRODUCTION AND BACKGROUND......................................................... 1

1.1 Rapid rates of neuronal conduction allow organisms to quickly adapt to their environment ......................................................... 1
1.2 Signal propagation along the axon: Importance of axon diameter and its effect on conduction velocity............................... 3
1.3 Neurofilament proteins, filament composition and post-translational modification................................................................. 6
1.4 Neurofilament are transported via slow axonal transport by motor proteins............................................................................. 11
1.5 The role of neurofilament phosphorylation in regulating neurofilament transport.............................................................. 14
1.6 Neurofilaments are determinants of radial growth in myelinated axons.................................................................................... 15
1.7 Maintenance of NF subunit stoichiometry is essential for radial axonal growth................................................................. 18
1.8 NF-M is the critical subunit for radial growth of myelinated axons......................................................................................... 19
1.9 Neurofilament C-terminal truncation revealed that NF-M C-terminus was required for radial growth in large myelinated axons........................................................................... 21
1.10 Phosphorylation of NF-M KSP repeats is not necessary for radial growth in myelinated axons............................................... 24
1.11 Thesis Synopsis..................................................................................................................................................... 25

CHAPTER 2 DISTAL TO PROXIMAL DEVELOPMENT OF PERIPHERAL NERVES REQUIRES THE EXPRESSION OF NEUROFILAMENT HEAVY........................................... 28
<table>
<thead>
<tr>
<th>Chapter</th>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Abstract</td>
<td>29</td>
</tr>
<tr>
<td>2.2</td>
<td>Introduction</td>
<td>30</td>
</tr>
<tr>
<td>2.3</td>
<td>Experimental Procedures</td>
<td>32</td>
</tr>
<tr>
<td>2.4</td>
<td>Results</td>
<td>33</td>
</tr>
<tr>
<td>2.5</td>
<td>Discussion</td>
<td>41</td>
</tr>
<tr>
<td>3.1</td>
<td>Abstract</td>
<td>46</td>
</tr>
<tr>
<td>3.2</td>
<td>Introduction</td>
<td>48</td>
</tr>
<tr>
<td>3.3</td>
<td>Experimental Procedures</td>
<td>51</td>
</tr>
<tr>
<td>3.4</td>
<td>Results</td>
<td>56</td>
</tr>
<tr>
<td>3.5</td>
<td>Discussion</td>
<td>70</td>
</tr>
<tr>
<td>4.1</td>
<td>Abstract</td>
<td>78</td>
</tr>
<tr>
<td>4.2</td>
<td>Introduction</td>
<td>79</td>
</tr>
<tr>
<td>4.3</td>
<td>Experimental Procedures</td>
<td>81</td>
</tr>
<tr>
<td>4.4</td>
<td>Results</td>
<td>90</td>
</tr>
<tr>
<td>4.5</td>
<td>Discussion</td>
<td>109</td>
</tr>
<tr>
<td>4.6</td>
<td>Appendix</td>
<td>113</td>
</tr>
<tr>
<td>5.1</td>
<td>Abstract</td>
<td>118</td>
</tr>
<tr>
<td>5.2</td>
<td>References</td>
<td>136</td>
</tr>
<tr>
<td>5.3</td>
<td>Vita</td>
<td>150</td>
</tr>
</tbody>
</table>
LIST OF TABLES

Table 3.1  Degenerate PCR primer design for amplification of NF-M Exon 3.... 52
Table 3.2  Number of NF-M KSP repeats among species.......................... 58
Table 3.3  NF-M exon 3 (C-terminus) Statistics........................................... 59
Table 3.4  Intraspecies sequencing from mouse, rat, and human individuals.... 60
Table 4.1  Myelin thickness and g-ratios were altered in axons of NF-M\textsuperscript{BovineTail} mice........................................................................ 108
Table 4.2  Reductions in myelin thickness were predicted to be sufficient to prevent increased motor nerve conduction velocity in NF-M\textsuperscript{BovineTail} mice...................................................... 110
LIST OF FIGURES

Figure 1.1 Cellular composition of a motor neuron........................................... 2

Figure 1.2 Increasing axonal diameter increases the efficiency of electrotonic conduction.......................................................... 5

Figure 1.3 Neurofilament subunits................................................................. 8

Figure 1.4 Neurofilaments subunits assemble to form 10 nm filaments......... 9

Figure 2.1 Radial growth of proximal axonal segments is most affected by loss of NF-H in sciatic nerve.................................................. 35

Figure 2.2 Reductions in axonal diameter occur along the length of the phrenic nerve.................................................................. 37

Figure 2.3 Neurofilament nearest neighbor distances are larger in proximal axonal segments in both wild type and NF-H−/− axons......... 40

Figure 3.1 Amplification of neurofilament medium (NF-M) Exon 3 by degenerate PCR reveals different lengths of NF-M C-terminal tail domains........................................................................... 57

Figure 3.2 Lysine–serine–proline (KSP) repeat number positively correlates with size in subset of mammals................................. 62

Figure 3.3 Mammalian phylogenies suggest independent neurofilament medium (NF-M) lysine–serine–proline (KSP) repeat expansion events across several clades..................................................... 63

Figure 3.4 Organization of lysine–serine–proline (KSP) repeats within the neurofilament medium (NF-M) C-terminal tail domain is conserved within, but not between, sub-groups of mammals....... 67

Figure 3.5 Expansion in the number of KSP repeats is correlated with larger axonal diameters............................................................. 71
Figure 4.1 Generation of an NF-M\textsuperscript{BovineTail} chimeric protein in mice by replacement of murine nefm exon3 with bovine exon....................... 91

Figure 4.2 Expression of NF-M\textsuperscript{BovineTail} had no effect on relative stoichiometries and accumulated levels of NF-L, NF-M, and NF-H but resulted in decreased NF-H phosphorylation...................... 93

Figure 4.3 Increasing the length of the NF-M C-terminus resulted in increased radial growth of large motor axons................................. 96

Figure 4.4 Expansion of NF-M C-terminus resulted in increased extension from the core of the filament.................................................. 99

Figure 4.5 Neurofilament spacing was unaffected in sensory and motor axons of NF-M\textsuperscript{BovineTail} mice......................................................... 103

Figure 4.6 Neurofilament clustering and density were unaffected in motor axons of NF-M\textsuperscript{BovineTail} mice............................................................. 107

Figure 4.7 Motor nerve conduction velocity and myelin structure was unaltered in NF-M\textsuperscript{BovineTail} mice.......................................................... 109

Figure 4.8 Sensory axon radial growth was unaffected in NF-M\textsuperscript{BovineTail} mice.................................................................................. 114

Figure 4.9 Neurofilament spacing was unaffected in sensory axons of NF-M\textsuperscript{BovineTail} mice................................................................. 116

Figure 5.1 Distal segments of axons develop prior to proximal segments due to delayed NF-H expression................................................ 124

Figure 5.2 The length of the NF-M C-terminus determines the magnitude of radial axonal growth......................................................... 131
MOLECULAR MECHANISMS OF RADIAL AXONAL GROWTH: INSIGHTS FROM ANALYSIS OF NEUROFILAMENT GENE-TARGETED MICE

DEVIN M. BARRY

ABSTRACT

Maturation of the peripheral nervous system requires establishment of axonal diameter which has a significant influence on the rate of signal propagation along the axon. Expansion of axonal diameter is referred to as radial growth and is dependent upon both myelination and neurofilaments. Neurofilaments (NFs) are the most abundantly expressed cytoskeletal proteins in myelinated axons and consist of neurofilament light (NF-L), medium (NF-M), and heavy (NF-H) subunits. NF-M and NF-H were hypothesized to regulate axonal diameter through myelin-dependent phosphorylation of lysine-serine-proline (KSP) repeats. Gene deletion of NF-L suggested that NF-L is required for assembly of NF subunits into mature NFs. NF-H gene deletion resulted in small reductions in axonal diameter suggesting that NF-H was dispensable for radial axonal growth. Gene deletion of NF-M and its carboxy terminus (C-terminus) resulted in substantial reductions in axonal diameter and reduced nerve conduction velocity. However, prevention of NF-M KSP phosphorylation did not
recapitulate the reduction in axonal diameter that was observed in NF-M C-terminal deleted mice. While NF-M was identified as the critical subunit and its C-terminus as the critical domain for radial axonal growth, KSP phosphorylation was not required. Therefore, the mechanism by which the NF-M C-terminus mediated radial axonal growth remained unresolved. It also remained unclear the role of NF-H expression in radial axonal growth.

Studies on radial axonal growth in NF-H gene deleted (NF-H−/−) were limited to only the most proximal axonal regions of peripheral nerves. To address the role of NF-H expression in radial growth of peripheral axons, I analyzed radial growth of axonal segments along the length of the phrenic nerve in NF-H−/− mice. The analysis indicated that axonal diameter was reduced along the entire length of the phrenic nerve with only the most distal segments being unaffected. NF number was reduced in the proximal axonal segments and was unaffected in the most distal segment of the phrenic nerve. Taking into consideration previous observations, the current analysis of NF-H−/− mice suggest that distal to proximal development of peripheral axons requires NF-H expression. Distal axonal segments underwent radial growth prior to proximal segments. As NF-H expression increases, NFs accumulate and radial growth occurs in more proximal axonal segments which proceeds radial growth in distal segments.

To better understand the function of the NF-M C-terminus in mediating radial axonal growth, I performed phylogenetic sequencing analysis of the NF-M C-terminus across several clades of mammals. Sequencing analysis suggested
that the C-terminus is divided into three sub-domains, two highly conserved regions flanking a highly variable region. Independent expansion events of the NF-M C-terminus occurred within multiple clades of mammals. All observed expansion events occurred by the addition of amino acids, including KSP repeats, in the variable sub-domain. NF-M C-terminal length correlated with axonal diameter in a subset of mammals. These data suggested that expansion of the NF-M C-terminus through addition of KSP repeats might have resulted in larger axonal diameters during mammalian evolution.

To further test whether expansion of the NF-M C-terminus increases axonal diameter, I performed gene replacement in mice to increase the length of the NF-M C-terminus. The endogenous NF-M C-terminus was replaced with the longer bovine NF-M C-terminus. Expression of the expanded NF-M C-terminus did not affect expression of NF-L or NF-H subunits and resulted in reduced NF-H phosphorylation. Increasing the length of the NF-M C-terminus increased radial axonal growth of large motor axons in mice. Despite increased axonal diameter, nerve conduction velocity was unaffected possibly due to a lack of increased myelin thickness. Failure of myelin to compensate suggest a lack of plasticity during the processes of myelination and radial axonal growth.

My dissertation has provided greater understanding of the mechanisms by which NF-H and NF-M regulate radial growth of axons. NF-H expression was developmentally delayed to facilitate distal to proximal radial growth of peripheral axons during postnatal development. Expansion of the NF-M C-terminus during...
mammalian evolution may have been a mechanism to increase radial axonal growth as larger mammals evolved. Expansion of the NF-M C-terminus in mice suggested that the length of the NF-M C-terminus determines the magnitude of radial growth in large motor axons.
CHAPTER 1
INTRODUCTION AND BACKGROUND

1.1. Rapid rates of neuronal conduction allow organisms to quickly adapt to their environment.

The function of the nervous system is to appropriately respond to external stimuli in a timely manner allowing organisms to adapt to their environment. For an organism to respond to external stimuli, sensory information from the environment must be internalized and processed eliciting an appropriate response. All of these processes typically occur within fractions of a second. Yet within this time frame, signals must be transmitted long distances from and to the periphery of the organism. It is, therefore, important that signals are transmitted rapidly for the nervous system to function appropriately. Thus, the functional speed of the nervous system allows for the organism to adapt to its environment quickly and increases the organism's chances of survival.

Neurons, which are the cells that make up the nervous system, are excitable cells that allow for signaling to occur over long distances. There are many types of neurons, and my research focuses primarily on motor neurons. Motor neurons convey information to the periphery resulting in movement through muscle contraction. A typical motor neuron (Fig. 1.1) is composed of a cell body that is located within the spinal cord. The cell body has small projections known as dendrites that receive input from other neurons. A cellular junction known as a synapse transmits the signal from the input, or presynaptic,
Figure 1.1. **Cellular composition of a motor neuron.** A motor neuron is composed of a cell body, which contains the nucleus (gray circle), and is located within the spinal cord. The cell body forms dendrites that receive input from other neurons. A motor neuron is also composed of a long projection known as an axon that connects the cell body to its prospective target, which are muscle fibers. Signals are transmitted along the axon to cause contraction of the muscle fibers. An axon connects to muscle fibers by synapses, which transmit signals from the motor neuron to the muscle fibers.
cell to the target, or postsynaptic, cell. A motor neuron is also composed of a long projection known as an axon. The axon typically functions as an output process for the neuron. When a motor neuron is excited by sufficient input from presynaptic cells, it triggers a signal that is sent along the axon to its postsynaptic target. The signal is then transmitted to the muscle through synapses at the axon terminal, referred to as the neuro-muscular junction, resulting in contraction of the muscle. Because motor neuron cell bodies are located within the spinal cord, motor axons must extend long distances to connect to their prospective target muscle fibers. Motor axons can be as long as 1 meter in humans and signals must be transmitted along the entire length for muscle contraction to occur. It is important that signals be transmitted rapidly along the axon for muscles to contract quickly and allow organisms to adapt to their environment with minimal delay.

1.2. Signal propagation along the axon: Importance of axon diameter and its effect on conduction velocity.

Signals are transmitted down the axon by the flow of current into the axon. Current flow into an axon results in a local change in the voltage across the axon membrane known as depolarization. As current enters the axon, the depolarizing event travels down the axon by the passive spread of charge, referred to as electrotonic conduction. The length constant (\(\lambda\)) is a measure of the efficiency of electrotonic conduction, and is the length at which the voltage change decays to ~37% of the initial membrane voltage change from the current source (Fig. 1.2).
The length constant varies directly with respect to membrane resistance and inversely with respect to internal axonal resistance, and both properties are related to the diameter of the axon (Hodgkin, 1947; Hodgkin and Rushton, 1946). While membrane resistance is largely determined by the number of ion leak channels in the membrane, axonal resistance is influenced greatly by axonal diameter (Hodgkin, 1947; Hodgkin and Rushton, 1946). Therefore, axons with larger diameters transmit signals faster (Gasser, 1950; Hursh, 1939; Rushton, 1951) due, in part, to more efficient electrotonic conduction.

Invertebrates, such as squid, have extremely large diameter axons, as large as 1 mm, that conduct rapidly (Hodes, 1953), but they have few peripheral axons relative to vertebrates such as mammals. Unlike squid, mammalian nerves are typically composed of thousands of individual axons. If increasing axonal diameter were the only mechanism to increase conduction velocity in mammals, axons would require diameters similar to those of the squid giant axon resulting in nerves that are prohibitively large for the mammalian nervous system. Mammals, therefore, required an alternative strategy to increase conduction rates while keeping the nervous system relatively small. Mammals evolved myelination, which is a process by which concentric layers of membrane are wrapped around the axon by Schwann cells in the peripheral nervous system. The wrapping of membrane layers provided insulation to the axon to prevent charge loss and also resulted in reducing capacitance along the myelinated region of axon. Prevention of charge loss and reduction in capacitance both
Figure 1.2. Increasing axonal diameter increases the efficiency of electrotonic conduction. Current flow into an axon results in a local change in the axon's voltage ($\Delta V_m$). The current flow, or charge, spreads passively from the input source (arrows) and decays with increasing distance from the input. Passive spread of charge is referred to as electrotonic conduction. The length constant ($\lambda$) is a measure of the efficiency of electrotonic conduction and varies directly with respect to membrane resistance and inversely with respect to internal axonal resistance. Both membrane and axonal resistance are influenced by axonal diameter. The length constant can be made effectively longer by increasing axonal diameter, which results in more efficient spread of charge down the axon.
contributed to increasing the rate of conduction in myelinated axons (Huxley and Stampfli, 1949; Koles and Rasinsky, 1972; Rasinsky and Sears, 1972). Myelination also initiated cell biological changes in axons that resulted in larger axonal diameters, which also contributed to increasing the rate of conduction (Hursh, 1939; Rushton, 1951; Waxman, 1980). Myelin-dependent expansion of axonal diameter is referred to as radial growth (Cleveland, 1996). Although the mechanism remains unknown, radial growth is dependent upon both myelination (de Waegh et al., 1992) and upon neurofilaments (NFs) (Ohara et al., 1993; Zhu et al., 1997).

1.3. Neurofilament proteins, filament composition and post-translational modification.

With the advent of the microscope in the 1800s, anatomists such as Santiago Ramon y Cajal, were the first to describe fibrous networks, which were named neurofibrils, within neurons. The development of the silver staining method in the late 1800s and early 1900s allowed for greater clarity of neurofibrils. Over 60 years later, electron microscopy revealed that neurofibrils were comprised of filaments ~10 nm in diameter and were thus named neurofilaments (Schmitt, 1968).

Neurofilaments (NFs) are type-IV intermediate filaments (IFs) (Fuchs and Weber, 1994), that are composed of neurofilament light (NF-L), medium (NF-M), and heavy (NF-H) subunits (Fig 1.3). Appearance of NF-L and NF-M subunits was detected during development as early as embryonic day 9 in mouse and
embryonic day 12 in rat (Carden et al., 1987; Cochard and Paulin, 1984). Appearance of the NF-H subunit was not detected until embryonic day 15 in rat and remained well below the levels of NF-L and NF-M until adulthood in rodents (Carden et al., 1987; Pachter and Liem, 1984; Shaw and Weber, 1982). In adults, NFs were the most abundant cytoskeletal proteins within myelinated axons (Lee and Cleveland, 1996). Mature NFs were obligate heteropolymers in vivo composed of NF-L, NF-M and NF-H (Ching and Liem, 1993; Lee et al., 1993) with NF-L forming dimers with NF-M or NF-H (Fig. 1.4). Formation of the classic 10 nm filament for NFs required the expression of NF-L (Lee et al., 1993). Analysis of purified bovine neurofilaments suggested a subunit ratio of ~4:2:1 (NF-L: NF-M: NF-H) (Scott et al., 1985). However, the composition of the NF filament varied with developmental stages as NF-H expression was delayed relative to NF-L and NF-M (Pachter and Liem, 1984; Shaw and Weber, 1982). More recent analysis suggested that NFs also contain a fourth type-IV IF subunit, α-internexin, in the mature central nervous system (Yuan et al., 2006).

All three neurofilament subunits contained an amino terminal (N-terminal) head domain, a central rod domain, and a carboxy terminal (C-terminal) tail domain (Fig. 1.3). The three domains had different affects on assembly of NFs into 10 nm filaments. The N-terminal head domain of NF-L promoted lateral association of protofilaments into protofibrils and ultimately the 10 nm filament (Fig. 1.4A) (Heins et al., 1993). The central rod domain of NF subunits consisted of a highly conserved ~310 amino acid α-helical domain containing a hydrophobic heptad repeat that was required for heterodimer formation and
Figure 1.3. Neurofilament subunits. Schematic of wild type mouse NF-L, NF-M, and NF-H subunits. The number 1 denotes the start of the amino terminus of each subunit. Like other intermediate filaments, NF subunits each contain an amino terminal head (orange), central rod (green), and carboxy terminal tail (violet) domain. The rod domain of each subunit contain α-helical regions that result in subunits forming coiled-coil dimmers. Unlike other intermediate filament, NF-M and NF-H have long tail domains that contain regions of lysine-serine-proline (KSP) (cyan) repeats.
Figure 1.4. Neurofilament subunits assemble to form 10 nm filaments. (A) Schematic of NF assembly into the classic 10 nm filament. NF subunits form polar heterodimers that assemble into half-staggered, anti-parallel strands to form apolar protofilaments. Two protofilaments polymerize to form protofibrils. Two to four protofibrils polymerize to form the 10 nm filament that is characteristic of intermediate filaments. (B) Schematic of NF fiber with side arm projections that are the carboxy terminal tail domains of NF-M and NF-H.
assembly into the 10 nm filament (Gill et al., 1990). The C-terminal domain of NF-L controlled the lateral association of protofibrils so that the NF assembly terminated as a 10 nm filament (Heins et al., 1993). Unlike other IFs, NF heteropolymers contained side arms that projected from the core of a mature 10 nm filament (Fig. 1.4B) (Hirokawa et al., 1984). The side arm projections were identified as the C-terminal tail domains of both the NF-M and NF-H subunits (Garcia et al., 2003; Hirokawa et al., 1984; Hisanaga and Hirokawa, 1988; Rao et al., 2002).

The amino acids of the N-terminal head domain of NFs contained potential sites for posttranslational modification, including multiple threonine and serine residues. Post-translational modification of subunit head domains influenced NF assembly (Dong et al., 1993; Sihag and Nixon, 1991; Wong and Cleveland, 1990). O-linked glycosylation sites were identified in the N-termini of all three NF subunits (Dong et al., 1993; Dong et al., 1996). Moreover, both NF-L (Sihag and Nixon, 1991) and NF-M (Sihag and Nixon, 1990) were serine phosphorylated within their N-terminal head domains. Mimicking constitutive phosphorylation, through mutation of serine 55 to asparate (NF-L$^{S55D}$) within the N-terminus of NF-L, resulted in aggregates of NF-L in transfected mammalian cells (Gibb et al., 1996). Furthermore, low-level expression of NF-L$^{S55D}$ transgene in mice resulted in NF accumulations within neuronal cell bodies (Gibb et al., 1998). Taken together, these data suggested that N-terminal phosphorylation of NF-L may alter NF organization.
The C-terminus of NF-M and NF-H also contained many potential sites for posttranslational modification. NF-M and NF-H C-termini contained central lysine-serine-proline (KSP) repeats that have four variants: KSP, KXSP, KXXSP and KSD (Julien et al., 1988; Lees et al., 1988; Levy et al., 1987; Myers et al., 1987). The serine residues of these highly conserved KSP repeats were phosphorylated in vivo (Julien and Mushynski, 1982; Julien and Mushynski, 1983; Xu et al., 1992). The C-terminal tail of NF-H also contained several O-linked glycosylation sites (Dong et al., 1996). Recently, O-linked glycosylation sites were identified within the C-terminus of NF-M (Deng et al., 2008; Ludemann et al., 2005). Evidence suggested that O-linked glycosylation of NF-M was synchronously regulated with NF-M phosphorylation (Deng et al., 2008). The role of post-translational modifications of NF KSP repeats in radial axonal growth remains unclear.

1.4. Neurofilaments are transported via slow axonal transport by motor proteins.

Proteins of neurons are primarily synthesized within the cell body. For NFs and other proteins localized within the axon, active transport must occur to send proteins along the entire length of the axon. To investigate transport along the axon, radiolabeled amino acids were injected into the cell bodies of neurons (Taylor and Weiss, 1965). Radiolabeled amino acids were incorporated into newly synthesized proteins within the cell bodies and subsequently transported along axons. After a period of time ranging from hours to months, nerves were
dissected and cut into segments for analysis of radioactive signal.

Pulse-chase radiolabeling of newly synthesized proteins revealed two rates of axonal transport, referred to as slow and fast transport, with the majority of the radioactive signal contained in the slow component of transport (McEwen and Grafstein, 1968). Five cytoskeletal proteins accounted for greater than 75% of the radiolabeled proteins from slow transport (Lasek, 1967). Three of the five proteins were identified as the three NF subunits (Hoffman and Lasek, 1975). Further analysis of slow transport in dorsal root ganglia (DRG) axons revealed that slow transport consists of two components, Slow component a and b, that transport different proteins (Oblinger and Lasek, 1985). Slow component a traveled at a rate of ~0.2-2.5 mm per day (Oblinger and Lasek, 1985), and consisted of five cytoskeletal proteins, including the three NF subunits, as previously observed (Hoffman and Lasek, 1975). Slow component b traveled at a rate of 2-8 mm per day (Willard et al., 1974), and consisted of numerous proteins including actin (Black and Lasek, 1979). Fast axonal transport consisted of the movement of membranous organelles at a rate of greater than 400 mm per day (Ochs, 1972).

Axonal transport occurs from the cell body down the axon, referred to as anterograde transport, and also occurs from the synapse toward the cell body, referred to as retrograde transport. Until recently, the mechanism by which cargoes were transported in slow anterograde and retrograde transport remained unclear. Cultured neuron analysis of NF transport using fluorescently labeled NFs suggested that the motor proteins used in fast axonal transport, kinesin and
dynein, also transported NFs in slow transport (Roy et al., 2000; Shah et al., 2000; Wang et al., 2000; Yabe et al., 1999). The instantaneous velocities of NFs were similar to that of fast transport by kinesin-based motors (Wang and Brown, 2001; Wang et al., 2000), but the overall transport rate was slowed due to prolonged pauses and frequent reversals in direction of motors transporting NFs (Roy et al., 2000; Uchida and Brown, 2004; Wang and Brown, 2001; Wang et al., 2000). Deletion of Kif5A, a kinesin heavy-chain motor subunit, in mice resulted in NF accumulation in cell bodies and a severe reduction in axonal diameter (Xia et al., 2003). Similarly, loss of dynein function, through over expression of dynamitin, inhibited retrograde transport as well as progressive degeneration of motor neurons (LaMonte et al., 2002).

Determining the mechanisms that regulate NF-motor protein association remain as key steps in understanding NF transport. Evidence suggested that NFs interacted directly with kinesin (Jung et al., 2005) and dynein (Wagner et al., 2004). Native NFs purified from spinal cords were associated with both kinesin and dynein and were transported bi-directionally (Shah et al., 2000). NF-kinesin association may be mediated by NF-M and NF-H subunits. NF-M was detectable in optic nerves in mice lacking both NF-L and NF-H (Hirokawa, 1997; Yuan et al., 2003). Moreover, loss of NF-M resulted in increased rates of NF-H and NF-L transport in both optic (Yuan et al., 2006) and sciatic nerve (Jacomy et al., 1999). Targeted deletion of NF-H resulted in increased transport rates for NF-M and NF-L within sciatic nerve (Zhu et al., 1998), and resulted in increase rates of transport for the leading edge of radiolabeled NF-M and NF-L in optic nerve (Rao
1.5. The role of neurofilament phosphorylation in regulating neurofilament transport.

NF-M and NF-H C-terminal KSP phosphorylation has long been thought to regulate NF transport (Lewis and Nixon, 1988). Evidence suggested that phosphorylation of NF-M and NF-H on KSP repeats resulted in accumulation of NFs during radial growth of myelinated axons (Sanchez et al., 1996; Sanchez et al., 2000). NF-H and NF-M KSP phosphorylation resulted in slowing of NF axonal transport in transfected neurons (Ackerley et al., 2003) and optic axons (Jung and Shea, 1999). Moreover, dephosphorylated NFs, recognized by SMI-32 antibody, were detected in proximal regions of axons, whereas NF phospho-epitopes, recognized by the RT97 antibody, were detected only in distal axonal regions in optic nerve (Nixon et al., 1994). NF accumulations coincided with the appearance of other phospho-epitopes, such as SMI-31 and SMI-34, primarily on NF-H in optic nerve (Sanchez et al., 2000). However, in the absence of NF-H, phosphorylation of NF-M was increased with the appearance of the RT97 epitope and accumulation of NFs in optic nerve (Sanchez et al., 2000). Evidence also indicated that NF phosphorylation resulted in decreased association of kinesin with NFs (Jung et al., 2005; Yabe et al., 2000). Taken together these data suggest that NF-M and NF-H KSP phosphorylation negatively regulated NF transport and resulted in local accumulation of phosphorylated NFs.

Recent evidence challenged the long-standing hypothesis of NF-M and
NF-H KSP phosphorylation in regulating NF transport. NFs purified from spinal cord were associated with both kinesin and dynein, and were transported bidirectionally along microtubules (Shah et al., 2000). When separated by SDS-PAGE, purified spinal cord NFs migrated with a similar mobility to NFs from crude homogenates (Shah et al., 2000). This suggested that purified NFs retained phosphorylation when co-purified with kinesin and dynein (Shah et al., 2000). Moreover, mice were generated that express C-terminally truncated NFs in which all identified phosphorylation sites were deleted (Rao et al., 2003; Rao et al., 2002). The rate of NF transport in optic nerve was unaltered in C-terminally truncated NF-M (Rao et al., 2003) or NF-H (Rao et al., 2002). Interestingly, loss of a single C-terminus resulted in compensatory phosphorylation of the other remaining NF C-terminus (Rao et al., 2003; Rao et al., 2002). Transport analyses in mice that express both NF-H and NF-M C-termini truncated proteins would be necessary to rule out compensatory phosphorylation maintaining normal rates of NF transport.

1.6. Neurofilaments are determinants of radial growth in myelinated axons.

Motor neurons undergo two distinct phases of growth with each phase dependent upon different components of the neuronal cytoskeleton. During development in utero, motor neurons extend axons from the ventral horn of the spinal cord to their prospective targets. This first phase of growth is dependent upon actin/myosin for growth cone motility and microtubules for timely delivery of proteins from the cell body and to provide direction to the growing axon terminus.
(Letourneau, 1996). After stable synapse formation, axons undergo a second distinctive phase of growth, known as radial growth (Cleveland, 1996). Radial growth initiates with, and is dependent upon, the formation of compact myelin resulting in axonal diameter increases of up to ten-fold in mice (de Waegh et al., 1992). During radial growth, neurofilaments (NFs) accumulate and become the most abundant cytoskeletal proteins (Cleveland, 1996), and the number of NFs correlate with axonal diameter (Friede and Samorajski, 1970; Hoffman et al., 1985b). More importantly, subunit composition of NFs is critical for radial growth (Xu et al., 1996).

It was hypothesized that the formation of compact myelin initiated NF KSP phosphorylation, which then regulated radial growth in myelinated axonal regions (Cleveland, 1996). Indeed, NF-M and NF-H C-termini were more heavily phosphorylated within myelinated axonal regions relative to unmyelinated regions (de Waegh et al., 1992; Hsieh et al., 1994; Starr et al., 1996; Yin et al., 1998). Moreover, axonal diameter was increased in myelinated axonal regions relative to unmyelinated regions of the same axon (de Waegh et al., 1992; Hsieh et al., 1994; Nixon et al., 1994; Yin et al., 1998). Taken together, these data suggested the myelin-dependent NF phosphorylation hypothesis of radial growth, in which myelination regulated NF phosphorylation which then influenced radial expansion of axons.

The first discovery that radial growth was dependent upon the axonal accumulation of NFs was not found in rodents but in birds, specifically the Japanese quail. Myelinated axons in both the central and peripheral nervous
systems of the quiver (Quv) quail were hypotrophic (Yamasaki et al., 1991) and axonal NFs were absent (Yamasaki et al., 1992; Yamasaki et al., 1991). The absence of axonal NFs was due to loss of NF-L expression through a spontaneous nonsense mutation in Japanese quail (Ohara et al., 1993), which resulted in axons that failed to grow radially in response to myelination (Ohara et al., 1993; Sakaguchi et al., 1993). Failure of radial growth resulted in reduced nerve conduction velocities (Sakaguchi et al., 1993), quivering and mild ataxia (Yamasaki et al., 1991). The dependence of radial growth on axonal NF accumulations was confirmed, through serendipity, by a transgenic mouse line that was originally produced to study NF-H distribution. The C-terminus of NF-H was replaced with the enzyme β-galactosidase (NF-H-LacZ) (Eyer and Peterson, 1994). Expression of the NF-H-LacZ transgene resulted in NFs that failed to transport into the axon resulting in axons that failed to grow radially in response to myelination (Eyer and Peterson, 1994). Expression of NF-H-LacZ also resulted in a significant decrease in survival of 4th lumbar (L4) motor axons in aged mice (Eyer and Peterson, 1994). While no overt phenotypes were observed in NF-H-LacZ mice, when challenged the mice were deficient in balance and equilibrium which suggested sensorimotor deficits (Dubois et al., 2002).

Targeted deletion of NF-L (NF-L<sup>−/−</sup>) in mice confirmed many of the results observed in both the Quv quail and NF-H LacZ mice. NFs were absent in sciatic nerve axons and axons failed to grow radially (Zhu et al., 1997). Nerve conduction velocity was also severely reduced (Kriz et al., 2000). Interestingly, compensatory changes in myelination did not occur in response to reduced radial
axonal growth, which resulted in abnormally thick myelin sheaths in NF-L−/− mice (Zhu et al., 1997). NF-L−/− mice also had a small, but significant, reduction in the survival of L5 motor axons (Zhu et al., 1997). Despite the significant reduction in both conduction velocity and motor axon number, no overt phenotypes were observed in NF-L−/− mice. However, as in NF-H-LacZ mice, NF-L−/− mice display abnormalities consistent with sensorimotor deficits when challenged (Dubois et al., 2005).

1.7. Maintenance of NF subunit stoichiometry is essential for radial axonal growth.

Expression of NF transgenes in cells lacking endogenous intermediate filaments suggested that mouse NF-L could not self-assemble into 10nm filaments in vivo but required the co-expression of NF-M or NF-H (Lee et al., 1993). Moreover, analysis of mature NFs extracted from peripheral nervous tissue indicated that NF subunit stoichiometry was maintained at ~4:2:1 ratio (NF-L:NF-M:NF-H) (Scott et al., 1985). To analyze the role of NF subunit stoichiometry in radial growth, transgenic mice were generated that over express NF subunits altering NF subunit stoichiometry in vivo. Interestingly, over expression of any of the three NF subunits individually in mice resulted in reduced radial growth of axons in the 5th lumbar (L5) motor root and abnormal NF accumulations in perikarya and proximal axonal regions (Cote et al., 1993; Wong et al., 1995; Xu et al., 1993; Xu et al., 1996). Conversely, over expression of NF-M or NF-H combined with NF-L over expression resulted in increased
radial growth of L5 motor axons (Xu et al., 1996). Over expression of NF-M alone resulted in a compensatory decrease of NF-H expression while NF-L levels remained unaltered (Wong et al., 1995; Xu et al., 1996). Likewise, over expression of NF-H alone led to compensatory decreases in NF-M expression with no change in NF-L expression (Xu et al., 1996) suggesting that NF-M and NF-H competed for association with NF-L during NF assembly. Taken together these results suggested that alterations in NF stoichiometry alone, regardless of the individual roles of each NF subunit, were sufficient to alter radial growth of myelinated axons.

1.8. NF-M is the critical subunit for radial growth of myelinated axons.

Since loss of NF-L expression through spontaneous mutation in Japanese quail (Ohara et al., 1993) or targeted deletion in mice (Zhu et al., 1997) resulted in axons without NFs, it remained unclear as to which subunit was required for radial growth. NF-M and NF-H C-termini were heavily phosphorylated within myelinated internodes (de Waegh et al., 1992; Hsieh et al., 1994; Nixon et al., 1994), with NF-H being phosphorylated to near stoichiometric levels on its KSP-serine residues (Julien and Mushynski, 1982; Julien and Mushynski, 1983). If KSP phosphorylation of NFs was required for radial growth, NF-H, the larger and more heavily phosphorylated subunit, was predicted to be the primary contributor. However, deletion of the NF-M and NF-H subunits gave unexpected results.

NF-M targeted deletion (NF-M\(^{-/-}\)) resulted in a significant decrease in radial growth (Elder et al., 1998a). Unlike wild type control mice that establish a
bimodal distribution of small and large diameter axons in the L5 motor root, NF-M^{+/−} mice formed a unimodal distribution of small axons (Elder et al., 1998a) similar to that observed in NF-L^{+/−} mice (Zhu et al., 1997). NF-L levels and overall NF content in sciatic nerve axons were reduced (Elder et al., 1998a), and NF transport rates were increased (Jacomy et al., 1999). Reductions in radial growth in regions of the CNS were also observed, and nerve conduction velocity was significantly reduced (Elder et al., 1998a; Kriz et al., 2000).

NF-H deletion in mice supported the role of NF-M requirement for radial growth. Three lines of gene deleted NF-H (NF-H^{+/−}) mice were generated in which particular alterations were observed in each line (Elder et al., 1998b; Rao et al., 1998; Zhu et al., 1998). The overall conclusion from all three NF-H^{+/−} lines of mice was that loss of NF-H resulted in a minor reduction in radial growth (Elder et al., 1998b; Rao et al., 1998). Despite the small reduction in radial growth, NF-H^{+/−} mice had significantly reduced nerve conduction velocity, which was similar to the observed reduction in NF-M^{+/−} mice (Kriz et al., 2000). Furthermore, refractory period of action potentials was prolonged and outward rectification was decreased in NF-H^{+/−} mice (Kriz et al., 2000) suggesting a role for NF-H in modulating ion channel function in large axons. Deletion of NF-H also resulted in significant increase in the rate of NF transport along the sciatic nerve (Zhu et al., 1998).

Interpretation of the NF-M^{+/−} and NF-H^{+/−} phenotypes was complicated by compensatory changes in remaining cytoskeletal components. Deletion of NF-M resulted in decreased NF-L levels and decreased NF-H phosphorylation in spinal
cord (Elder et al., 1998a). NF-H deletion resulted in a two-fold increase in NF-M expression (Rao et al., 1998), increased phosphorylation of NF-M (Zhu et al., 1998), and increased axonal microtubule content (Rao et al., 1998; Zhu et al., 1998). Taken together the analysis of NF-M\(^{-/-}\) and NF-H\(^{-/-}\) mice suggested NF-M as the critical subunit for radial growth. However, alterations in NF subunit stoichiometry were previously demonstrated to be sufficient to reduce radial growth (Xu et al., 1996). Thus it remained difficult to conclude which subunit, NF-M, NF-H or both, was important for radial axonal growth through classic targeted deletion.

1.9. Neurofilament C-terminal truncation revealed that NF-M C-terminus was required for radial growth in large myelinated axons

Traditional gene deletions of individual NF subunits were complicated by altered NF subunit stoichiometry and compensatory changes in remaining cytoskeletal components. To directly understand the role of NF C-termini in radial growth, gene targeted mice were generated that express C-terminally truncated NF-H (Garcia et al., 2003), NF-M (Garcia et al., 2003; Rao et al., 2002), or both truncated NF-M and NF-H forms (Garcia et al., 2003). Unlike the complete gene deletions that altered subunit composition, NF-H and NF-M C-terminally truncated mice display normal NF subunit stoichiometry. Gene targeting was first performed on NF-H (NF-H\(^{tailΔ}\) mice), resulting in the loss of 612 amino acids including all 51 KSP repeat phosphorylation sites. The truncation of NF-H resulted in only minor axonal changes. Wild-type mice displayed a typical
bimodal distribution of L5 motor axon diameters with a smaller and larger peak at ~1 and ~6 µm, respectively (Garcia et al., 2003). At age 2 months, NF-H$^{\text{tail}\Delta}$ mice displayed small reductions in radial growth of both the smaller and larger groups of L5 motor axons relative to wild type (Garcia et al., 2003; Rao et al., 2002). Yet by 6 months of age, the peaks of L5 motor axon diameters of NF-H$^{\text{tail}\Delta}$ mice were indistinguishable from wild type for both smaller and larger axonal populations (Garcia et al., 2003; Rao et al., 2002). Based upon the 2-month and 6-month axonal diameter distribution data, the NF-H C-terminus did not affect the final size of myelinated motor axons but influenced the rate of radial growth.

Unlike NF-H, NF-M C-terminal truncation resulted in substantial axonal alterations. Truncation of NF-M resulted in the loss of 426 amino acids including all 7 KSP repeat sites from the C-terminus (NF-M$^{\text{tail}\Delta}$ mice) (Rao et al., 2003). At age 2 months, NF-M$^{\text{tail}\Delta}$ mice displayed a small reduction in radial growth of the smaller group of L5 motor axons similar to that in NF-H$^{\text{tail}\Delta}$ mice. However, NF-M$^{\text{tail}\Delta}$ mice displayed a much more significant reduction in radial growth of the larger group of L5 motor axons compared to both NF-H$^{\text{tail}\Delta}$ mice and wild-type mice (Garcia et al., 2003). Unlike NF-H$^{\text{tail}\Delta}$ mice, NF-M$^{\text{tail}\Delta}$ mice at 6 months had severely reduced radial growth of the large motor axons. Moreover, NF-H C-terminal phosphorylation was increased in NF-M$^{\text{tail}\Delta}$ mice, but did not compensate for the loss of the NF-M C-terminus and its phosphorylation in determining axonal diameter (Garcia et al., 2003). The reductions in radial growth of the large motor axons in NF-M$^{\text{tail}\Delta}$ mice resulted in ~30% reduction in sciatic nerve conduction velocity at 6 months (Garcia et al., 2003).
The generation of mice in which both the NF-M and NF-H C-termini were deleted (NF-(M/H)\textsuperscript{tailΔ} mice) confirmed the roles of both C-termini (Garcia et al., 2003). NF-(M/H)\textsuperscript{tailΔ} mice at 2 months displayed the most severe reduction in peak radial growth of the large L5 motor axons compared to NF-M\textsuperscript{tailΔ} and NF-H\textsuperscript{tailΔ} mice (Garcia et al., 2003). However, at 6 months NF-(M/H)\textsuperscript{tailΔ} mice had axon diameter distributions that were nearly identical to NF-M\textsuperscript{tailΔ} mice (Garcia et al., 2003). These findings supported previous results that suggested NF-H C-terminus affected the rate of radial growth, while the NF-M C-terminus determined the diameter of large motor axons.

To determine whether NF spacing was affected by the loss of NF C-termini, nearest neighbor distances (NND) between NFs were measured within large motor axons of NF-H\textsuperscript{tailΔ}, NF-M\textsuperscript{tailΔ} and NF-(M/H)\textsuperscript{tailΔ} mice. Deletion of the NF-H C-terminus in NF-H\textsuperscript{tailΔ} mice did not affect NND of NFs, and did not affect overall radial growth (Garcia et al., 2003). At 6 months, NNDs in NF-M\textsuperscript{tailΔ} mice were reduced relative to wild type mice, and NNDs were further reduced in NF-(M/H)\textsuperscript{tailΔ} mice relative to NF-M\textsuperscript{tailΔ} mice (Garcia et al., 2003). However, at 6 months, axonal diameters of NF-M\textsuperscript{tailΔ} and NF-(M/H)\textsuperscript{tailΔ} mice were indistinguishable (Garcia et al., 2003). Taken together, the NND and diameter data suggested that NF spacing and radial growth were not directly correlated in myelinated axons.

Analysis of NF C-terminally truncated mice demonstrated that NF-M and its C-terminus were required for radial growth, which was consistent with the myelin dependent NF phosphorylation hypothesis. Truncation of the NF-M C-
terminus removed all KSP phosphorylation sites suggesting that NF-M phosphorylation was necessary for radial growth.

1.10. **Phosphorylation of NF-M KSP repeats was not necessary for radial growth in myelinated axons.**

To provide a more direct test of the NF phosphorylation hypothesis of radial growth, site-directed mutagenesis was utilized to mutate all KSP repeat serine codons to alanine codons (NF-M^{S\rightarrow A} mice). Mutation to alanine codons was predicted to prevent phosphorylation of KSP repeats (Julien and Mushynski, 1982; Julien and Mushynski, 1983) without deleting all 426 amino acid codons of the NF-M C-terminus. The mutation of all seven KSP repeat serines to alanines resulted in increased electrophoretic mobility of NF-M protein (Garcia et al., 2009), which was similar to the increase in mobility observed after prolonged treatment of NF-M with alkaline phosphatase (Julien and Mushynski, 1982). The expression of NF-M^{S\rightarrow A} in mice had no affect on other cytoskeletal protein levels including NF-H and NF-L (Garcia et al., 2009).

Radial growth of L5 motor root axons was measured at 2 and 6 months in both NF-M^{S\rightarrow A} mice and wild type littermates. The peaks in axonal diameter of both small and large axons in NF-M^{S\rightarrow A} mice were indistinguishable from wild type littermates at both time points (Garcia et al., 2009). There were more axons at 7-8µm in NF-M^{S\rightarrow A} mice at 2 months (Garcia et al., 2009). The increase in large motor axons resulted in a small, but significant, difference in the overall diameter distributions between NF-M^{S\rightarrow A} and wild type mice at 2 months.
(Garcia et al., 2009). Additionally, at 2 months, the total number of L5 motor axons was reduced relative to wild type (Garcia et al., 2009). However, by 6 months, axonal diameter distributions and number of axons within L5 motor roots of NF-M<sup>S→A</sup> mice were essentially indistinguishable from wild type mice (Garcia et al., 2009). As expected, due to similar sized axons, nerve conduction velocities were indistinguishable from wild type (Garcia et al., 2009). Phosphorylation of NF-M KSP repeats was not necessary for myelin dependent radial growth of axons.

To determine whether NF spacing was affected by NF-M KSP phosphorylation, NNDs between NFs were measured within large motor axons of NF-M<sup>S→A</sup> mice. NF NNDs were reduced in NF-M<sup>S→A</sup> mice at 2 and 6 months despite no changes in radial growth of axons (Garcia et al., 2009). This data supported the previous findings in NF-M<sup>tailΔ</sup> and NF-(M/H)<sup>tailΔ</sup> mice (Garcia et al., 2003) that NF spacing and radial growth do not correlate directly in myelinated axons.

Taken together, these observations made in NF-M<sup>S→A</sup> mice suggested that phosphorylation of NF-M KSP repeats was not necessary for myelin dependent radial growth of axons. Therefore, NF-M C-terminus mediated radial axonal growth by a mechanism that was independent of NF KSP phosphorylation.

1.11. Thesis Synopsis

Specification of axonal diameter is a key component of motor neuron function as it is a major axonal property that specifies the velocity of signal
conduction. Within myelinated axons, the diameter of the internode is increased relative to the diameter of unmyelinated axonal segments, which is necessary for rapid impulse transmission. Neurofilaments (NFs) are the major cytoskeletal proteins of myelinated axons, and are phosphorylated on conserved lysine-serine-proline repeats (KSP repeats) along their carboxy termini within myelinated regions of an axon.

For 17 years, phosphorylation of NF C-termini was hypothesized to be necessary for radial axonal growth. Deletion of NF-L suggested that NF-L was required for NFs to assemble and that radial growth was dependent upon axonal accumulation of NFs (Ohara et al., 1993; Zhu et al., 1997). NF-M deletion in mice suggested that NF-M was the critical subunit for radial growth (Elder et al., 1998a). Deletion of NF-H resulted in only a minor reduction in radial growth (Elder et al., 1998b; Rao et al., 1998; Zhu et al., 1998) suggesting a potentially different role for NF-H in radial growth. Truncation of the NF-M C-terminus reduced peak radial axonal growth by ~40%, which resulted in ~30% reduction in the rate of conduction (Garcia et al., 2003). However, prevention of NF-M phosphorylation in NF-M^{S\rightarrow A} mice failed to recapitulate reduced radial growth of motor axons observed in mice expressing C-terminally truncated NF-M (Garcia et al., 2003; Garcia et al., 2009). Thus, generation of gene-targeted mice disproved the long-standing hypothesis, which predicted that myelin-dependent NF phosphorylation was necessary for radial axonal growth. Therefore, the role of NF-H and the mechanism by which NF-M regulated radial axonal growth remained unclear.
The goal of my thesis was to provide insight into the potential role of NF-H and to provide insight into the mechanism of NF-M mediated radial axonal growth. NF-H^{−/−} mice were generated 13 years ago yet no further studies have been performed to better understand its role in radial growth. I analyzed NF-H^{−/−} mice to better understand the role of NF-H in proximal and distal development of peripheral nerve fibers. To better understand the function of NF-M in radial growth, phylogenetic analysis of the NF-M C-terminus was performed across mammals, which resulted in the generation of a new hypothesis for NF-M mediated radial axonal growth. Moreover, I directly tested my new hypothesis through the development of a novel line of gene-targeted mice.
CHAPTER 2

DISTAL TO PROXIMAL DEVELOPMENT OF PERIPHERAL NERVES REQUIRES THE EXPRESSION OF NEUROFILAMENT HEAVY

(This work was published in Neuroscience, 2010, Vol. 170, p. 16–21)

Hailian Shen¹*, Devin M. Barry¹,²* and Michael L. Garcia¹,²

¹C.S. Bond Life Sciences Center, ²Division of Biological Science University of Missouri, Columbia, MO 65211

* Authors contributed equally
2.1 Abstract.

At the initiation of radial growth, neurofilaments are likely to consist primarily of neurofilament light and medium as neurofilament heavy expression is developmentally delayed. To better understand the role of neurofilament heavy in structuring axons, axonal diameter and neurofilament organization were measured in proximal and distal segments of the sciatic nerve and along the entire length of the phrenic nerve. Deletion of neurofilament heavy reduced axonal diameters and neurofilament number in proximal nerve segments. However, neurofilament spacing was greater in proximal versus distal phrenic nerve segments. Taken together, these results suggest that loss of neurofilament heavy reduces radial growth in proximal axonal segments by reducing the accumulation of neurofilaments. As neurofilament heavy expression is developmentally delayed, these results suggest that without neurofilament heavy, the neurofilament network is established in a distal to proximal gradient perhaps to allow distal axonal segments to develop prior to proximal segments.

**Keywords:** neurofilament, development, radial growth, nerve conduction velocity, myelination
2.2 Introduction.

Specification of axonal diameter is a key component of neuronal function as it is one major axonal property that influences the rate of impulse propagation along the axon (Waxman, 1980). Radial growth, the process by which axonal diameter is established in peripheral nerves, is dependent upon neurofilaments (NFs) (Ohara et al., 1993; Zhu et al., 1997). NFs are obligate heteropolymers, and can be composed of neurofilament light (NF-L), medium (NF-M), heavy (NF-H) (Lee et al., 1993) and a-internexin (Yuan et al., 2006). However, the composition of the heteropolymer varies according to developmental stage. Expression of NF subunits is differentially regulated (Carden et al., 1987; Pachter and Liem, 1984; Shaw and Weber, 1982). NF-L and NF-M expression is detected as early as embryonic day 12 (E12) in rat (Carden et al., 1987) and E9-9.5 in mouse (Cochard and Paulin, 1984). In contrast, NF-H expression is delayed relative to NF-L and NF-M (Carden et al., 1987; Pachter and Liem, 1984; Shaw and Weber, 1982) with appreciable expression levels first observed at E15 in rat (Carden et al., 1987). However, these levels remained well below NF-L and NF-M levels until 3 months in rat (Pachter and Liem, 1984; Shaw and Weber, 1982).

Radial growth is also dependent upon the formation of compact myelin (de Waegh et al., 1992). Myelination begins around birth and can take several weeks to complete (Jessen and Mirsky, 1999). At the initiation of myelination, NFs within the axon are likely composed of NF-L and NF-M as NF-H expression is significantly reduced at this stage. As myelination progresses NFs become the most abundant structural protein in myelinated axons (Lee and Cleveland, 1996).
NF phosphorylation increases within myelinated axonal regions (de Waegh et al., 1992; Yin et al., 1998). Axonal diameter of myelinated axonal regions increases (de Waegh et al., 1992). Additionally, during myelination, NF-H expression increases (Pachter and Liem, 1984; Shaw and Weber, 1982). However, NF-H expression remains below the expression levels of NF-L and NF-M well after the completion of myelination (Shaw and Weber, 1982).

Radial growth and myelination initiates prior to significant accumulation of NF-H in axons. Gene targeting of NFs have established that NF-M (Elder et al., 1998a) and NF-L (Zhu et al., 1997) are required for radial growth as loss of each of these subunits results in small caliber, unimodal axons. Alternatively, NF-H was not required for radial growth in the majority of myelinated axons. Deletion of NF-H resulted in a bimodal distribution of axons with a slight reduction in axonal diameters of only the largest myelinated axons (Elder et al., 1998b; Rao et al., 1998; Zhu et al., 1998). However, deletion of NF-H resulted in increased rates of transport of NF-L and NF-M (Zhu et al., 1998). Interestingly, NF transport slows as development progresses (Hoffman et al., 1983). Temporally, developmental slowing of NF transport coincides with increasing expression of NF-H (Hoffman et al., 1983; Shaw and Weber, 1982). Moreover, radial growth is associated with slowing of NF transport (Hoffman et al., 1985a; Hoffman et al., 1984; Hoffman et al., 1985b). Collectively, these results suggest that at the initiation of myelination and radial growth axonal NFs are composed of NF-L and NF-M. As radial growth progresses, NF-H expression increases resulting in reduced rates of NF transport in the largest myelinated axons.
Previous analysis of NF-H deleted mice has focused on proximal axonal segments from 1-4 months (Elder et al., 1998b; Rao et al., 1998; Zhu et al., 1998). To determine whether there may be an interaction between NF content and transport that influences radial growth from proximal to distal, for the first time we examined the affects of NF-H loss along the length of multiple nerves.

2.3 Experimental Procedures.

**Mice breeding:** All animal protocols were approved by the University of Missouri Animal Care and Use Committee. NF-H−/− mice were a generous gift provided by Dr. Don W. Cleveland. Mice were housed in microisolator cages on a 12-h light/dark cycle and were given food and water *ad libitum*. NF-H deleted mice are maintained as homozygous deleted mice. Age matched C57Bl6 mice were used as controls.

**Tissue Preparation, Morphological Analysis:** NF-H−/− and C57Bl6 control mice were perfused intracardially with 2.5% glutaraldehyde and 4% formaldehyde in 0.1 M Sorenson’s phosphate buffer, pH 7.2, and post-fixed overnight in the same buffer. Fifth lumbar (L5) ventral roots, the motor branch of the peroneal nerve and phrenic nerves (proximally at the exit point from the spinal canal, distally at the point entry into diaphragm) were dissected out. Phrenic nerves were dissected into equal length segments and sequentially named as first (1st), second (2nd), third (3rd) and fourth (4th) segment from the distal to proximal ends. Samples were treated with 2% osmium tetroxide, washed, dehydrated, and
embedded in Epon-Araldite resin as previously described (Garcia et al., 2009). Thick sections (0.75 µm) for light microscopy were stained with p-phenylene diamine. Cross sections of L5 motor axons, peroneal and phrenic nerves were analyzed from four to five mice per genotype and age group. Axonal diameters were measured using the AxioVision Digital Image Processing Software (Carl Zeiss MicroImaging). Entire roots were imaged, imaging thresholds were selected individually, and the cross sectional area of each axon was calculated and reported as a diameter of a circle of equivalent area. Axon diameters were grouped into 0.5 µm bins. Means, for total number of axons and neurofilaments, were analyzed for statistical significance using unpaired Student’s *t*-test for wild type versus NF-H−/− mice. Bimodal distributions of motor axon diameter distributions were analyzed for overall statistical significance using Mann–Whitney *U* test. All statistic tests were performed using Sigmaplot 11 software (Systat Software Inc.). Differences were considered significant if *P* value < 0.05.

Thin sections (60–90 nm) were cut from prepared resin blocks with a Leica Ultracut E ultramicrotome, stained with 1% aqueous uranyl acetate for 15 min followed by lead salts for 2 min. Images of selected neurons were collected at 80 kV with a JEOL 1200FX at the indicated magnification of 3000. Neurofilaments were traced and nearest-neighbor distance calculations made.

2.4 Results.

Radial axonal growth is reduced in proximal segments of motor axons from NF-H−/− mice
Previous analysis of motor axons in NF-H<sup>−/−</sup> mice suggested that loss of the NF-H subunit affected radial growth primarily in the largest axons (Elder et al., 1998b; Rao et al., 1998; Zhu et al., 1998). To determine the longitudinal affect of deleting NF-H on axons, we have examined diameter of all axons in proximal, 5<sup>th</sup> lumbar ventral root, and distal, motor branch of the peroneal nerve, segments of the sciatic nerve (Figure 2.1A and C). Analysis of proximal segments of sciatic nerve indicates that the largest fibers are affected by deletion of NF-H at 5 months (Figure 2.1A). The peak diameter of the largest fibers is reduced in NF-H<sup>−/−</sup> lumbar roots relative to wild type control (Figure 2.1A). The differences in axonal diameter distributions in proximal axonal segments were statistically significant. Deletion of NF-H is associated with reduced survival of motor and sensory axons in the fifth lumbar motor and sensory roots (Rao et al., 1998). However, in our analysis there was no difference in the number of axons in wild type versus NF-H<sup>−/−</sup> proximal nerve segments (Figure 2.1B). Moreover, two other previous studies of NF-H<sup>−/−</sup> mice did not report any loss of motor axons (Elder et al., 1998b; Zhu et al., 1998).

Diameter distributions in distal segments of sciatic nerve were unimodal in both wild type and NF-H<sup>−/−</sup> mice (Figure 2.1C). There was no difference in the peak diameter obtained by wild type or NF-H<sup>−/−</sup> mice (Figure 2.1C). Analysis of the total population of axons by Mann-Whitney U test indicates that there is a statistically significant difference in the distribution of axonal diameters in the peroneal nerve. This change maybe due to decreased large motor axons in distal segments. In proximal axonal segments, NF-H<sup>−/−</sup> axons contribute 18% to the
Figure 2.1. Radial growth of proximal axonal segments is most affected by loss of NF-H in sciatic nerve. Axonal diameter distributions of proximal (A) and distal (C) segments of sciatic nerve from 5-month-old wild type and NF-H−/− mice. Points represent the averaged distribution of axon diameters from the entire roots of three to five mice for each genotype. Vertical dashed lines indicate peaks in axonal populations. Axonal populations were analyzed for overall statistical differences utilizing the Mann–Whitney U-test. There was a significant difference in diameter distributions between wild type and NF-H−/− axons populations (L5 Roots \( P < 0.001 \), peroneal nerve \( P < 0.001 \)). Number of axons in L5 motor roots (B) and the motor branch of the peroneal nerve (D) of 5-month-old wild type and NF-H−/− mice. Counts are the average from three to five mice for each genotype. Means, for total axon counts, were analyzed for statistical differences using Student’s \( t \)-test.
total number of axons within the range of 6-9 µm whereas NF-H⁻/⁻ axons contribute 35% to the total population of axons within the range of 2-8 µm in distal segments. This difference in diameter distributions cannot be attributed to differences in axon number, as there was no difference in the total number of axons between wild type and NF-H⁻/⁻ mice (Figure 2.1D).

These results suggest that deletion of the NF-H subunit has a greater affect on peak diameter distribution of large axons within proximal axons of the sciatic nerve. However, our analysis was performed in the 5th lumbar motor root and the motor branch of the peroneal nerve. Without tracing, it is not possible to determine the percentage of overlap between the two populations within the analyzed nerve regions. To ensure proximal and distal axonal segments represent a single population of axons, we analyzed the long, unbranched region of the phrenic nerve. The phrenic nerve was dissected and sectioned into four equal length segments with segment 1 being most distal from the spinal cord (Figure 2.2A). Qualitatively, deletion of NF-H resulted in reduced axonal diameters in proximal nerve segments (segment 4). Loss of NF-H did not alter the number of axons in phrenic nerve for any of the segments. For simplicity, we have only shown total axon numbers for segment 1 and 4 (Figure 2.2B and C). The diameter of all axons within each phrenic nerve segment was examined in 5-month-old wild type and NF-H⁺/⁺ mice (Figure 2.2D-G). All diameter distributions were unimodal in both wild type and NF-H⁺/⁺ mice. In the most distal segment, segment 1, diameter distributions are indistinguishable for wild type and NF-H⁺/⁺ mice (Figure 2.2D). However, diameter distributions for NF-H⁻/⁻ mice start to
Figure 2.2. Reductions in radial growth occur along the length of the phrenic nerve. Cross sections of axonal profiles from four segments of equal length of the entire phrenic nerve of 5-month-old wild type and NF-H^{−/−} mice (A). The four segments are sequentially named as first (1st), second (2nd), third (3rd) and fourth (4th) segment from the distal to proximal ends. Scale Bar, 5 µm. (B and C) Number of axons at (B) 1st and (C) 4th segments of the phrenic nerve of wild type and NF-H^{−/−} mice at 5 months of age. Counts are the average from four to five mice for each genotype. Means, for total axon counts, were analyzed for statistical differences using Student’s t-test.
**Figure 2.2 Cont’d. Reductions in radial growth occur along the length of the phrenic nerve.** (D–G) Diameter distributions of axons of the (D) 1st, (E) 2nd, (F) 3rd, and (G) 4th segments of the phrenic nerve in 5-month-old wild type and NF-H$^{-/-}$ mice. Points represent the averaged distribution of axon diameters from the entire peroneal nerve motor branch of four to five mice for each genotype. Vertical dashed lines indicate peaks in axonal populations. Axonal populations were analyzed for overall statistical differences utilizing the Mann–Whitney $U$-test. There was a significant difference in diameter distributions between wild type and NF-H$^{-/-}$ axons populations for 2nd, 3rd and 4th segments of the phrenic nerve ($P=0.1$ for Segment 1; $P<0.001$ for Segments 2–4).
become smaller in segments closer to the spinal cord. In segments 2 and 3, the peak diameter is reduced by 0.5 µm in NF-H<sup>−/−</sup> mice (Figure 2.2E and F). In the most proximal segment, segment 4, peak diameter in NF-H<sup>−/−</sup> mice is reduced 1.5 µm relative to wild type mice (Figure 2.2G). Loss of NF-H led to an alteration in the longitudinal profile of the axons. Wild type axons are larger as they exit the spinal cord becoming smaller as they approach their targets. The peak diameter for wild type mice is reduced 0.5 µm as axons approach their target (Figure 2.2D and G). However, axons in NF-H<sup>−/−</sup> mice are the smallest as they exist the spinal cord (Figure 2.2G) increasing as they approach their targets. NF-H<sup>−/−</sup> axonal diameters are 1 µm larger near their targets versus near the spinal cord.

**Neurofilament number is reduced while nearest neighbor distances are larger in proximal axonal segments of NF-H<sup>−/−</sup> phrenic nerves**

Deletion of NF-H resulted in a slight decrease in neurofilament (NF) spacing within axons of the sciatic nerve (Rao et al., 1998). We analyzed electron micrographs of wild type and NF-H<sup>−/−</sup> mice from all segments of the phrenic nerve (data not shown). Qualitatively there did not appear to be large alterations in NF number or organization within segments 2 and 3 (data not shown). Therefore, our analysis focused on segments 1 and 4 (Figure 2.3A). NF spacing was determined for segments 1 and 4 in both wild type and NF-H<sup>−/−</sup> axons. For segment 1, the peak in nearest neighbor distances was similar in wild type versus NF-H<sup>−/−</sup> axons (Figure 2.3A). However, NF-H<sup>−/−</sup> axons appear to have more NFs that are spaced at and below 27 nm whereas wild type axons have...
Figure 2.3. **Neurofilament nearest neighbor distances are larger in proximal axonal segments in both wild type and NF-H<sup>−/−</sup> axons.** Distributions of nearest neighbor distances of neurofilaments from axons of the 1st and 4th segments of the phrenic nerve in 5-month-old wild type and NF-H<sup>−/−</sup> mice (A). Vertical dashed lines indicate peaks in neurofilament populations. Number of neurofilaments in axons from the 1st (B) and 4th (C) segments of the phrenic nerve in 5-month-old wild type and NF-H<sup>−/−</sup> mice. Neurofilament number is significantly reduced in Segment 4 of NF-H<sup>−/−</sup> axons relative to wild type Segment 4 and NF-H<sup>−/−</sup> Segment 1. Neurofilament numbers were analyzed for statistical significance using unpaired Student’s t-test. * P< 0.05.
more NFs that are spaced between 34-51 nm (Figure 2.3A). Moreover, there were no differences in the total number of NFs in wild type versus NF-H−/− in segment 1 (Figure 2.3B). Analysis of NF spacing in segment 4 resulted in a much different profile than segment 1 (Figure 2.3A). While there was an obvious peak in NF nearest neighbor distance in segment 1 (27 nm), there were several peaks in the distribution profiles for segment 4 with the highest number of NFs being spaced 34 nm apart (Figure 2.3A). Overall the nearest neighbor distance profiles were similar between wild type and NF-H−/− NFs in segment 4. Interestingly, the total number of NFs was significantly reduced in NF-H−/− axons relative to control (Figure 2.3C). There were differences in NF distributions and NF numbers between wild type and NF-H−/− axons. Moreover, interesting differences appear when comparing segment 1 to segment 4 within a genotype. In wild type axons, NF spacing is different between segment 1 and 4 (Figure 2.3A upper versus lower panel). This difference in spacing was also noted in NF-H−/− axons (Figure 2.3A upper versus lower panel). However, NF-H−/− axons contain more NFs that are spaced at or below 27 nm. Additionally, NF number is significantly reduced in segment 4 versus segment 1 in NF-H−/− axons, which is not the case in wild type axons.

2.5 Discussion.

Our current analysis of NF-H−/− axons may begin to offer insight into the developmental regulation of NF-H expression and the role of NF-H in radial growth by tying together several distinct observations. During development, NF-H
expression, in rat, remains well below the level of NF-L and NF-M until 3 months
(Pachter and Liem, 1984; Shaw and Weber, 1982). Therefore, during the early
period of radial growth, it is likely that axonal NFs consist primarily of NF-L and
NF-M. As NF-H expression increases, radial growth of the largest axons
becomes dependent upon NF-H (Figure 2.1A). Dependence on NF-H is apparent
in both proximal and distal segments. However, radial growth is reduced to a
greater extent in proximal axonal segments. We also examined the phrenic nerve
to determine if NF-H is required in proximal segments. Analysis of the phrenic
nerve indicates that proximal segments of nerves are dependent upon NF-H
expression for radial growth. Furthermore, the most distal segments of the
phrenic nerve do not require NF-H expression (Figure 2.2G). Taken together
these data suggest that proximal segments require NF-H expression for radial
growth.

Deletion of NF-H results in NFs that are composed entirely of NF-L and
NF-M. A consequence of NF-H deletion is that even during later stages of
development NF-H\(^{-/}\) axons contain NFs that are similar in composition to axonal
NFs at early stages of development when NF-H expression is significantly below
the expression of NF-L and NF-M. Therefore, NF composition is “frozen” at an
earlier developmental stage. If NF-H\(^{-/}\) mice serve as a suitable model for early
development, then NF-H expression may be delayed to allow more distal
segments to develop prior to proximal segments. Mechanistically, distal
segments may develop prior to proximal segments due to increased NF content
(Figure 2.3C and D), as NF content is a major determinant of axonal diameter in
myelinated fibers (Friede and Samorajski, 1970; Hoffman et al., 1984; Sanchez et al., 1996). Taken together our results suggest that early in development when NF-H expression is low, NF accumulation and therefore radial growth of axons occurs in a distal to proximal manner.

NF-H expression begins to significantly increase between postnatal day 10 and 3 months of age with the most rapid rate of increase occurring between postnatal day 24 and 3 months (Shaw and Weber, 1982). As NF-H expression increases, NF transport rates would likely begin to decrease (Willard and Simon, 1983; Zhu et al., 1998). Slowing of NF transport is associated with developmental progression (Hoffman et al., 1983), and it coincides temporally with increased NF-H expression (Hoffman et al., 1983; Shaw and Weber, 1982). The highest rate of slowing in NF transport occurred between 3 and 10 weeks of age (Hoffman et al., 1983), which corresponds to the time when NF-H expression is dramatically increasing (Shaw and Weber, 1982). Moreover, radial growth is associated with slowing of axonal transport (Hoffman et al., 1985a; Hoffman et al., 1984; Hoffman et al., 1985b). Interestingly, NF content, NF spacing and radial growth were similar in wild type and NF-H−/− axons at 5 months in distal segments of the phrenic nerve. However, proximal segments had reduced NF content, NF spacing and radial growth. Taken together, these data suggest that increased NF-H expression slows axonal transport allowing proximal accumulation of NFs and radial growth.

Our analysis of both proximal and distal segments of both sciatic and phrenic nerves have given us new insights that may tie together many
observations made regarding NF-H. We propose that NF-H expression remains low during development to allow distal segments of nerves to develop prior to proximal segments. Increasing NF-H expression as development precedes functions to reduce the rate of NF transport allowing proximal accumulations of NFs resulting in radial growth.
CHAPTER 3

VARIATION OF THE NEUROFILAMENT MEDIUM KSP REPEAT SUB-DOMAIN ACROSS MAMMALIAN SPECIES: IMPLICATIONS FOR ALTERING AXONAL STRUCTURE

(This work was published in the *Journal of Experimental Biology*, 2010, Vol. 213, p.128-136)

Devin M. Barry\(^1,2\), Cory Carpenter\(^1,2\), Craig Yager\(^1,2\), Ben Golik\(^1,2\), Kyle J. Barry\(^1,2\), Hailian Shen\(^1,2\), Oliver Mikse\(^3\), Lori S. Eggert\(^1\), David J. Schulz\(^1\), and Michael L. Garcia\(^1,2\)

\(^1\)Department of Biological Sciences, \(^2\)Bond Life Sciences Center, University of Missouri-Columbia, Columbia, MO 65211, \(^3\)Department of Pharmacology, Pennsylvania State University College of Medicine, Hershey, PA 17033
3.1. Abstract:
The evolution of larger mammals resulted in a corresponding increase in peripheral nerve length. To ensure optimal nervous system functionality and survival, nerve conduction velocities were likely to have increased to maintain the rate of signal propagation. Increases of conduction velocities may have required alterations in one of the two predominant properties that affect the speed of neuronal transmission: myelination or axonal diameter. A plausible mechanism to explain faster conduction velocities was a concomitant increase in axonal diameter with evolving axonal length. The carboxy terminal tail domain of the neurofilament medium subunit is a determinant of axonal diameter in large caliber myelinated axons. Sequence analysis of mammalian orthologs indicates that the neurofilament medium carboxy terminal tail contains a variable lysine-serine-proline (KSP) repeat sub-domain flanked by two highly conserved sub-domains. The number of KSP repeats within this region of neurofilament medium varies among species. Interestingly, the number of repeats does not change within a species, suggesting that selective pressure conserved the number of repeats within a species. Mapping KSP repeat numbers onto consensus phylogenetic trees reveals independent KSP expansion events across several mammalian clades. Linear regression analyses identified three subsets of mammals, one of which shows a positive correlation in the number of repeats with head-body length. For this subset of mammals, we hypothesize that variations in the number of KSP repeats within neurofilament medium carboxy
terminal tail may have contributed to an increase in axonal caliber, increasing nerve conduction velocity as larger mammals evolved.

Keywords: Neurofilaments, Conduction Velocity, Radial Growth, Microsatellite
3.2. Introduction: 

Establishing Nerve Conduction Velocity in Vertebrates

Neurons communicate through propagation of action potentials along the axon to their targets. Current flow into an axon results in a local change in the axon's voltage. As current enters the axon, this local depolarizing event propagates down the axon by the passive spread of charge from the input source, referred to as electrotonic conduction. The conduction velocity of action potentials down the axon is related to the length constant ($\lambda$), which varies directly with respect to membrane resistance ($R_m$), and inversely with respect to axial resistance ($R_a$) (Hodgkin, 1947; Hodgkin and Rushton, 1946). While $R_m$ is largely determined by the number of leak channels in the membrane, $R_a$ is influenced strongly by axonal diameter (Hodgkin, 1947; Hodgkin and Rushton, 1946). Therefore, axons with larger diameters have faster conduction velocities (Hursh, 1939; Rushton, 1951) due, in part, to more efficient electrotonic conduction via reduction of $R_a$. Indeed, invertebrates have large rapidly conducting axons (Hodes, 1953). However, mammalian nerves consist of hundreds of individual axons. If increasing axonal diameter were the only mechanism to increase conduction velocity, axons would require extremely large diameters resulting in nerves that are prohibitively large for the vertebrate nervous system. The evolution of myelin resulted in increased conduction velocity in relatively small axons.

While myelination increases conduction rates by preventing charge loss, it also induces cell biological changes in axons so that they expand their diameter,
which increases the rate of electrotonic conduction (Huxley and Stampfli, 1949). Myelin-dependent expansion of axonal diameter is referred to as radial axonal growth. Although the mechanism remains unknown, radial axonal growth is dependent upon both the formation of compact myelin (de Waegh et al., 1992) and upon the axonal accumulation of neurofilaments (NFs) (Ohara et al., 1993; Zhu et al., 1997). Within axons, NFs are responsible for establishing and maintaining the three dimensional array of axoplasm. Therefore, if mammals were under selective pressure to increase neuronal conduction velocities, then it is probable that NF cytoskeletal architecture, myelination, or both, were likely the evolutionary targets.

**Neurofilaments Are Determinants of Nerve Conduction Velocity**

NFs are type-IV intermediate filaments (IFs) (Fuchs and Weber, 1994), composed of neurofilament light (NF-L), neurofilament medium (NF-M), and neurofilament heavy (NF-H) subunits. Mature NFs are obligate heteropolymers composed of NF-L, NF-M and NF-H (Ching and Liem, 1993; Lee et al., 1993) with an ~4:2:1 (NF-L: NF-M: NF-H) stoichiometric ratio (Scott et al., 1985). Recent work suggests that NFs also contain a fourth type-IV IF subunit, α-internexin, in the mature central nervous system (Yuan et al., 2006). Formation of the classic 10 nm filament, characteristic of all IFs, requires the expression of NF-L (Lee et al., 1993). Unlike other IFs, NF heteropolymers have side arms that project from the core of a mature 10 nm filament (Hirokawa et al., 1984). These side arms, which contain a lysine-serine-proline (KSP) repeat region (Lees et al.,
1988; Myers et al., 1987), are the carboxy terminal (C-terminal) tail domains of both the NF-M and NF-H protein subunits (Hirokawa et al., 1984; Hisanaga and Hirokawa, 1988; Rao et al., 2003; Rao et al., 2002). The C-terminal 426 amino acid tail of the NF-M subunit is a determinant of axonal diameter in large caliber myelinated axons (Garcia et al., 2003; Rao et al., 2003). Furthermore, loss of this 426 amino acid C-terminal tail domain in mice results in a 30% reduction in sciatic nerve conduction velocity (Garcia et al., 2003).

The NF-M KSP Repeat Region: A Molecular Mechanism for Expanding Axonal Caliber and Increasing Nerve Conduction Velocity

The NF-M gene is composed of three exons and two introns (Levy et al., 1987; Myers et al., 1987). 22 amino acids of the end of the coiled-coil rod domain and all 426 amino acids of the C-terminal tail required for radial axonal growth are generated from exon 3 (Garcia et al., 2003). Sequence analysis of NF-M exon 3 identified a repeated KSP motif, within the C-terminal 426 amino acid tail in mouse (Levy et al., 1987; Myers et al., 1987; Napolitano et al., 1987). Short tandem repeat regions, such as microsatellites, are found throughout the genomes of most vertebrates (Schlotterer, 2004). While most are thought to be inconsequential to normal gene function, recent evidence indicates that alterations in short tandem repeat lengths may have contributed to the evolution of species-specific traits. Variations of repeats within cis-regulatory elements may lead to morphological changes in the evolution of species, due to alterations in gene regulation (Carroll, 2000). Moreover, sequence analyses of several
developmentally important genes in dog suggested that alterations in repeat lengths might have robust effects on dog morphology (Fondon and Garner, 2004). For example, alterations in the number of tandem repeats coding for stretches of glutamines and alanines within the Runx-2 gene correlated with changes in snout length and bending of the snout (Fondon and Garner, 2004). Contraction of a microsatellite within the Alx-4 gene was correlated with polydactyly in one breed of dog (Fondon and Garner, 2004). Similar to tandem repeat variations affecting snout morphology in dogs (Fondon and Garner, 2004), it is possible that expansion in the number of KSP repeats evolved as part of a mechanism to increase axonal diameter with increasing axonal length. Since the NF-M C-terminal tail is a determinant of axonal diameter in large caliber motor axons (Garcia et al., 2003), expansion in the number of KSP repeats within this region of NF-M may have been an evolutionary mechanism for increasing nerve conduction velocities to maintain the rate of signal propagation as larger mammals evolved.

3.3. Experimental Procedures:

Genomic DNA isolation, PCR amplification and Sequence Analysis

*Homo sapiens sapiens* (human) purified DNA from an ethnic diversity panel was obtained through European Collection of Cell Cultures (ECACC). DNA from four inbred strains of *mus musculus* (mouse) was isolated from tail biopsies from existing mice. Wild trapped mice tail snips were obtained from specimens collected in northwest and northeast Missouri. *Phoca vitulina* (harbor
seal) DNA was isolated from lymphoblasts that were provided as a gift from Dr. Mark Milanick. Tissues from Castor canadensis (beaver), Sciurus carolinensis (gray squirrel), Sus scrofa (feral hog), Ovis aries (sheep), Synaptomys cooperi (southern bog lemming), and Blarina hylophaga (short-tailed shrew) were obtained from the vertebrate collection at the University of Missouri. 

Hydrochaeris hydrochaeris (capybara) DNA was provided as a gift from Dr. Juan Campos of Kansas State University. Dr. Guangping Gao of the University of Massachusetts Medical School provided Pan troglodytes (chimpanzee), Macaca mulatta (rhesus macaque) and Macaca fascicularis (crab-eating macaque) DNA as a gift. The San Diego Zoological Society provided DNA from their Frozen Zoo® collection for Loxodonta africana africana (savanna elephant), Giraffa camelopardalis rothschildi (giraffe), Ceratotherium simum simum (Southern white rhinoceros), Hippopotamus amphibious kiboko (hippopotamus), and Choloepus hoffmani (Hoffman’s two-toed sloth). DNA was isolated from all tissues by standard phenol-chloroform extraction.

Degenerate primers were designed for NF-M exon 3 based on published sequences (Table 3.1). In these sequences, the NF-M protein ends with either the two amino acids Serine and Aspartate (SD) or Glycine and Aspartate (GD).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF-M Exon 3 Degenerate Forward</td>
<td>5'-AAACTMCTRGGGGKGAAGAGACYAGAT-3'</td>
</tr>
<tr>
<td>NF-M Exon 3 Degenerate Reverse</td>
<td>5'-CTGGGTGACTTCCCTTKACWATGGCRTGTGAAG-3'</td>
</tr>
</tbody>
</table>

M= A or C, R= A or G, K= G or T, Y= C or T
To minimize primer complexity, codons for these amino acids were omitted from the reverse degenerate primer. The forward degenerate primer was designed to the beginning of NF-M exon 3. The approximate locations of exon 3 degenerate PCR primers are indicated in a schematic of NF-M C-terminus (Fig. 3.1A).

Amplification using PCR was performed using 50-100ng of genomic DNA, 10 or 20pmol each of forward and reverse primer, 2.5 units of Ex Taq (Takara), Taq Polymerase (Invitrogen), or GoTaq (Promega), 1X of manufacturer’s buffer, 50-100 µM of each deoxyribonucleotide triphosphate in a total volume of 25 or 50 µL. PCR reactions were performed using the following parameters: one cycle of 95°C denaturation (5 min); 35 cycles of 95°C denaturation (30 s), 55-60°C annealing (30 s), and 72°C extension (2 min); one cycle of 72°C final extension (5 min). 10% of each reaction was analyzed by agarose gel electrophoresis to confirm amplification. PCR products were in the range of 1.3-1.6 kb (Fig. 3.1B).

The remaining PCR products were purified using either Qiagen PCR purification kits or the Qiagen Gel Extraction kits. Purified products were sequenced using the forward and reverse PCR primers, as well as specific internal primers (data not shown). DNA sequencing was performed on an ABI 3730 capillary DNA analyzer utilizing Big Dye Terminator chemistry at the DNA core facility at the University of Missouri. Analysis of sequencing was performed using DNAStar programs (DNAStar, Inc.). The previously published sequences of human, *Oryctolagus cuniculus* (rabbit), *Equus caballus* (horse), *Bos taurus* (cow), *Canus lupus familiaris* (dog), and *Rattus norvegicus* (Norway rat) were obtained from GenBank (Benson et al., 2009). The BLAST tool at the Ensemble Genome
Browser was used to identify NF-M exon 3 from *Gorilla gorilla* (gorilla), *Tursiops truncatus* (bottlenose dolphin), and *Pteropus vampyrus* (large flying fox (bat)) reference assemblies (Hubbard et al., 2009). The ClustalW (Slow/Accurate Gonnet) method was used in all protein alignments in MegAlign (DNASTar, Inc.) (Thompson et al., 1994).

**Phylogenetic and Statistical Sequence Analysis**

Since the "KLLEGEE" codons marked the beginning of exon 3 and are highly conserved across all intermediate filaments, we determined the reading frame by identifying this motif within our DNA sequences. We used this sequence to convert DNA sequences to protein sequences for phylogenetic analysis. NF-M exon 3 protein sequences were aligned in Megalign (DNASTar, Inc.) (Thompson et al., 1994). The ClustalW method aligned the sequences using the Slow-Accurate pairwise alignment parameters with a gap penalty of 10.00 and gap length of 0.10. The ClustalW method also used a Gonnet 250 protein weight matrix in the alignment. Construction of consensus trees were performed by MegAlign v.8.0 (DNASTar, Inc.) using the Neighbor Joining Method (Saitou and Nei, 1987) with 10,000 bootstrap trials and a random number generator seed of 111.

An established consensus tree of placental mammalian phylogeny was adapted (Murphy et al., 2001) to map NF-M KSP repeats and highlight independent KSP expansion events. Only species that were sequenced for NF-M exon 3 were indicated and species without sequencing were omitted for
simplicity. Branching points for omitted species were collapsed as well for simplicity. Species that were sequenced for NF-M exon 3 but were not used to generate the phylogeny were incorporated at appropriate points based on other mammalian phylogenies (Bininda-Emonds et al., 2007) and were indicated by dashed lines.

To compare NF-M KSP number with animal size, linear regression analysis of KSP numbers with head-body length (Macdonald, 1984; Myers, 2006) were performed using Sigmaplot v.11 (Systat Software Inc.). The PredictProtein Server was used to predict secondary structure of the NF-M tail domain alignment (Rost et al., 2004).

**Axonal Quantification and Diameter Distributions**

Mice and rats were perfused intracardially with 4% formaldehyde in 0.1 M Sorenson's phosphate buffer, pH 7.2, and post-fixed overnight. The 5th lumbar root was isolated as previously described (Garcia et al., 2003).

Cows were sacrificed by lethal injection of pentobarbital into the carotid artery. The 5th lumbar root was dissected and immediately submerged in 4% paraformaldehyde in 0.1 M Sorenson's phosphate buffer, pH 7.2. The tissue was allowed to fix overnight at 4°C. Samples were treated with 2% osmium tetroxide, washed, dehydrated, and embedded in Epon-Araldite resin. Thick sections (0.75 μm) for light microscopy were stained with toluidine blue. Axonal diameters were measured using the AxioVision Digital Image Processing Software (Carl Zeiss)
Axon diameters were grouped in 0.5-µm bins as previously described (Garcia et al., 2003).

3.4. Results:

Variation in the NF-M Tail Domain Across Mammalian Species

We amplified (Fig. 3.1A) and sequenced the NF-M C-terminal tail from species representing several Orders of the Class Mammalia. The alignment of translated DNA sequences revealed two regions of high sequence identity flanking a highly divergent KSP repeat region. Based on amino acid percent identity and insertion events among mammals, the NF-M tail domain is divided into three sub-domains: the amino, KSP repeat, and carboxy sub-domains (Fig. 3.1B). After sequencing, we quantified and classified the KSP repeats of all mammalian species analyzed (Table 3.2). Additionally, the NF-M C-terminal tail domain amino acids were abundant in glutamate and lysine. Amino acid compositions and overall charges of NF-M C-termini were similar across mammals (Table 3.3). Structural analysis of the C-terminal tail domain failed to predict any obvious secondary structure (Rost et al., 2004).

No Allelic Variation of NF-M KSP repeats

Previously, sequence analysis of the NF-H C-terminal tail determined that intraspecies variations in the number of KSP repeats occurred in both dogs (Green et al., 2005), and humans
Figure 3.1. Amplification of neurofilament medium (NF-M) Exon 3 by degenerate PCR reveals different lengths of NF-M C-terminal tail domains. (A) NF-M exon 3, which codes for the entire C-terminal tail domain, was amplified from genomic DNA utilizing degenerate primers. PCR product sizes were estimated by direct comparison with λ DNA Hind III Digest and ΦX174 Hae III Digest molecular weight markers (New England Biolabs Inc., Ipswich, MA, USA) after electrophoresis on 1XTAE 1% agarose gels. NTC = no template control. (B) Schematic of NF-M protein illustrating the head, rod and tail domains. The C-terminal tail domain is highlighted showing the approximate location of degenerate primer annealing sites and the three sub-domains of the NF-M tail domain, with percentage identities for each sub-domain.
Table 3.2. Number of NF-M KSP repeats among species.

<table>
<thead>
<tr>
<th>Species</th>
<th>KSP</th>
<th>KXSP</th>
<th>KXXSP</th>
<th>KSD</th>
<th>Total</th>
<th>NCBI Accession #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hippopotamus</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>5</td>
<td>FJ427306</td>
</tr>
<tr>
<td>White Rhinoceros</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>5</td>
<td>FJ427310</td>
</tr>
<tr>
<td>Bottlenose Dolphin</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>5</td>
<td>Ensembl Reference Assembly</td>
</tr>
<tr>
<td>Horse</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>5</td>
<td>NW_001867404</td>
</tr>
<tr>
<td>Large Flying Fox (Bat)</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>5</td>
<td>Ensembl Reference Assembly</td>
</tr>
<tr>
<td>Southern Bog Leanning</td>
<td>4</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>6</td>
<td>FJ468475</td>
</tr>
<tr>
<td>Rabbit</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>6</td>
<td>P54938</td>
</tr>
<tr>
<td>Two-toed Sloth</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>6</td>
<td>FJ427309</td>
</tr>
<tr>
<td>Beaver</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>6</td>
<td>FJ427300</td>
</tr>
<tr>
<td>Dog</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>6</td>
<td>XP_543237</td>
</tr>
<tr>
<td>Harbor Seal</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>6</td>
<td>FJ427305</td>
</tr>
<tr>
<td>Short-tailed Shrew</td>
<td>4</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>7</td>
<td>FJ468476</td>
</tr>
<tr>
<td>Mouse</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>7</td>
<td>NP_032717</td>
</tr>
<tr>
<td>Rat</td>
<td>5</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>8</td>
<td>P12839</td>
</tr>
<tr>
<td>Eastern Gray Squirrel</td>
<td>3</td>
<td>0</td>
<td>7</td>
<td>1</td>
<td>11</td>
<td>FJ427308</td>
</tr>
<tr>
<td>Crab-eating macaque</td>
<td>9</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>11</td>
<td>FJ810221</td>
</tr>
<tr>
<td>Chimpanzee</td>
<td>11</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>13</td>
<td>FJ668668</td>
</tr>
<tr>
<td>Capybara</td>
<td>10</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>14</td>
<td>FJ427301</td>
</tr>
<tr>
<td>Rhesus macaque</td>
<td>13</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>15</td>
<td>FJ668669</td>
</tr>
<tr>
<td>Gorilla</td>
<td>13</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>15</td>
<td>Ensembl Reference Assembly</td>
</tr>
<tr>
<td>Human</td>
<td>13</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>15</td>
<td>NP_005373</td>
</tr>
<tr>
<td>Feral Hog</td>
<td>3</td>
<td>8</td>
<td>7</td>
<td>1</td>
<td>19</td>
<td>FJ427303</td>
</tr>
<tr>
<td>Giraffe</td>
<td>14</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>19</td>
<td>FJ427304</td>
</tr>
<tr>
<td>Elephant</td>
<td>15</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>19</td>
<td>FJ427302</td>
</tr>
<tr>
<td>Sheep</td>
<td>16</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>21</td>
<td>FJ427307</td>
</tr>
<tr>
<td>Cow</td>
<td>17</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>22</td>
<td>O77788</td>
</tr>
</tbody>
</table>
### Table 3.3. NF-M exon 3 (C-terminus) Statistics

<table>
<thead>
<tr>
<th>Species</th>
<th>Residues</th>
<th>Neutral Sites</th>
<th>Anionic Sites (-)</th>
<th>Cationic Sites (+)</th>
<th>KSP Phosphorylation Sites(^a)</th>
<th>Total Charge when Dephosphorylated</th>
<th>Total Charge when Phosphorylated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hippopotamus</td>
<td>461</td>
<td>229</td>
<td>146</td>
<td>86</td>
<td>5</td>
<td>-60</td>
<td>-70</td>
</tr>
<tr>
<td>White Rhino</td>
<td>461</td>
<td>237</td>
<td>141</td>
<td>83</td>
<td>5</td>
<td>-58</td>
<td>-68</td>
</tr>
<tr>
<td>Dolphin</td>
<td>456</td>
<td>230</td>
<td>139</td>
<td>87</td>
<td>5</td>
<td>-52</td>
<td>-62</td>
</tr>
<tr>
<td>Horse</td>
<td>451</td>
<td>226</td>
<td>144</td>
<td>81</td>
<td>5</td>
<td>-63</td>
<td>-72</td>
</tr>
<tr>
<td>Flying Fox (Bat)</td>
<td>447</td>
<td>235</td>
<td>132</td>
<td>80</td>
<td>6</td>
<td>-52</td>
<td>-64</td>
</tr>
<tr>
<td>Lemming</td>
<td>443</td>
<td>220</td>
<td>142</td>
<td>81</td>
<td>6</td>
<td>-61</td>
<td>-73</td>
</tr>
<tr>
<td>Rabbit</td>
<td>457</td>
<td>234</td>
<td>140</td>
<td>83</td>
<td>6</td>
<td>-57</td>
<td>-69</td>
</tr>
<tr>
<td>Two-toed Sloth</td>
<td>452</td>
<td>237</td>
<td>137</td>
<td>78</td>
<td>6</td>
<td>-59</td>
<td>-71</td>
</tr>
<tr>
<td>Beaver</td>
<td>459</td>
<td>235</td>
<td>141</td>
<td>83</td>
<td>6</td>
<td>-58</td>
<td>-70</td>
</tr>
<tr>
<td>Dog</td>
<td>460</td>
<td>229</td>
<td>148</td>
<td>83</td>
<td>6</td>
<td>-65</td>
<td>-77</td>
</tr>
<tr>
<td>Harbor Seal</td>
<td>457</td>
<td>228</td>
<td>146</td>
<td>83</td>
<td>6</td>
<td>-63</td>
<td>-75</td>
</tr>
<tr>
<td>Short-tailed Shrew</td>
<td>464</td>
<td>236</td>
<td>147</td>
<td>81</td>
<td>7</td>
<td>-66</td>
<td>-80</td>
</tr>
<tr>
<td>Mouse</td>
<td>448</td>
<td>223</td>
<td>145</td>
<td>80</td>
<td>7</td>
<td>-65</td>
<td>-79</td>
</tr>
<tr>
<td>Rat</td>
<td>445</td>
<td>222</td>
<td>143</td>
<td>80</td>
<td>8</td>
<td>-63</td>
<td>-79</td>
</tr>
<tr>
<td>Gray Squirrel</td>
<td>504</td>
<td>254</td>
<td>157</td>
<td>93</td>
<td>11</td>
<td>-64</td>
<td>-86</td>
</tr>
<tr>
<td>Crab-eating macaque</td>
<td>490</td>
<td>256</td>
<td>145</td>
<td>89</td>
<td>11</td>
<td>-56</td>
<td>-78</td>
</tr>
<tr>
<td>Chimpanzee</td>
<td>501</td>
<td>263</td>
<td>147</td>
<td>91</td>
<td>13</td>
<td>-56</td>
<td>-82</td>
</tr>
<tr>
<td>Capybara</td>
<td>510</td>
<td>270</td>
<td>144</td>
<td>96</td>
<td>14</td>
<td>-48</td>
<td>-76</td>
</tr>
<tr>
<td>Rhesus macaque</td>
<td>517</td>
<td>271</td>
<td>151</td>
<td>95</td>
<td>15</td>
<td>-56</td>
<td>-86</td>
</tr>
<tr>
<td>Gorilla</td>
<td>513</td>
<td>275</td>
<td>144</td>
<td>94</td>
<td>15</td>
<td>-50</td>
<td>-80</td>
</tr>
<tr>
<td>Human</td>
<td>514</td>
<td>271</td>
<td>148</td>
<td>95</td>
<td>15</td>
<td>-53</td>
<td>-83</td>
</tr>
<tr>
<td>Feral Hog</td>
<td>532</td>
<td>282</td>
<td>152</td>
<td>98</td>
<td>19</td>
<td>-53</td>
<td>-91</td>
</tr>
<tr>
<td>Giraffe</td>
<td>509</td>
<td>272</td>
<td>141</td>
<td>96</td>
<td>19</td>
<td>-54</td>
<td>-92</td>
</tr>
<tr>
<td>Elephant</td>
<td>549</td>
<td>283</td>
<td>157</td>
<td>109</td>
<td>19</td>
<td>-48</td>
<td>-86</td>
</tr>
<tr>
<td>Sheep</td>
<td>517</td>
<td>278</td>
<td>144</td>
<td>95</td>
<td>21</td>
<td>-49</td>
<td>-91</td>
</tr>
<tr>
<td>Cow</td>
<td>524</td>
<td>280</td>
<td>147</td>
<td>97</td>
<td>22</td>
<td>-50</td>
<td>-94</td>
</tr>
</tbody>
</table>

\(^a\) The charge valence of each KSP phosphorylation site changes from 0 to -2 when phosphorylated
(Al-Chalabi et al., 1999; Figlewicz et al., 1993; Tomkins et al., 1998; Vechio et al., 1996), albeit loss of KSP repeats in NF-H are associated with motor neuron disease (Al-Chalabi et al., 1999; Green et al., 2005). To determine if intraspecies variation in the number of KSP repeats occurred within the NF-M tail domain, exon 3 of the NF-M gene was sequenced from 127 mice (Table 3.4). These mice consisted of three inbred mouse strains (C57Bl6, FVB and CD1) and 10 wild-trapped mice. Moreover, 11 out-bred rats, and 76 humans, derived from an ethnic diversity panel, were analyzed (Table 3.4). Sequence analysis revealed no intraspecies allelic variations in the number of KSP repeats in any of the analyzed species (Table 3.4), suggesting stabilizing selection. Moreover, no amino acid variations were identified within exon 3 of any of the species, with the exception of published single nucleotide polymorphisms (SNPs) in mouse (NCBI SNPs rs31130946, rs30628515, rs30748232, rs31130043, rs52626242). No human SNPs were observed, but previous studies have observed rare SNPs within human NF-M exon 3 (Garcia et al., 2006; Vechio et al., 1996).

**Table 3.4. Intraspecies sequencing from mouse, rat, and human individuals.**

<table>
<thead>
<tr>
<th>Species</th>
<th>KSP repeat number</th>
<th>No. of individuals sequenced</th>
<th>Allelic Variation of KSPs Within NF-M tail domain</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD1 Mice (Inbred)</td>
<td>7</td>
<td>41</td>
<td>None</td>
</tr>
<tr>
<td>FVB Mice (Inbred)</td>
<td>7</td>
<td>21</td>
<td>None</td>
</tr>
<tr>
<td>C57/Bl6 Mice (Inbred)</td>
<td>7</td>
<td>55</td>
<td>None</td>
</tr>
<tr>
<td>Wild Mice</td>
<td>7</td>
<td>10</td>
<td>None</td>
</tr>
<tr>
<td>Rat (Outbred)</td>
<td>8</td>
<td>11</td>
<td>None</td>
</tr>
<tr>
<td>Human (Ethnic Diversity DNA Panel)</td>
<td>15</td>
<td>76</td>
<td>None</td>
</tr>
</tbody>
</table>
Correlations of NF-M KSP Number with Mammalian Size

We performed linear regression analyses of NF-M KSP repeat number with head-body length of each species (Fig. 3.2). Linear regression analyses indicated a bimodal distribution of species. The upper distributions (Fig. 3.2) suggested a positive correlation of NF-M KSP repeat number with mammalian size. However, the lower distributions (Fig. 3.2) suggested a weak negative correlation of NF-M KSP repeat number with mammalian size. The $R^2$ value was calculated from the linear regression of the red-grouped species only.

Mammalian Phylogenies Suggest Independent KSP Expansion Events Across Several Clades

The NF-M C-terminal sequences generated a consensus tree that resulted in similar phylogeny to the adapted placental mammalian tree (Fig. 3.3A and B). Although there were differences in superordinal branching between the trees, grouping of animal Orders appeared accurate in the NF-M tree as members of Rodentia, Primates, Artiodactyla, Carnivora, and Perissodactyla were monophyletic (Fig. 3.3B). Moreover, based upon ancestral lineage NF-M KSP expansion appears to have occurred independently several times within mammals. If we assume that mammalian ancestors of present-day mammals had fewer KSP repeats, then expansion of KSP repeats occurred across several clades. For example, dolphin, feral hog, hippo, giraffe, cow, and sheep are all members of the Superorder Cetartiodactyla, yet they diverge into four separate clades. Dolphin and hippo are more closely related and retained the ancestral
Figure 3.2. Lysine–serine–proline (KSP) repeat number positively correlates with size in subset of mammals. Linear regression analyses of KSP repeat numbers with head-body length of species resulted in a bimodal distribution of mammals. Three apparent groups were identified through this analysis: (1) mammals with a positive correlation in size versus KSP repeat number (red), (2) mammals with a negative or no correlation between size and KSP repeat number (green), and (3) mammals that can be classified in either group (blue). Red line is the best-fit line for only the red species group. Green line is the best-fit line for only the green species group. Significant $R^2$ value for the red line is indicated.
A

Placental Mammalian Phylogeny

<table>
<thead>
<tr>
<th>Animal</th>
<th>NF-M KSP Repeats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dolphin</td>
<td>5</td>
</tr>
<tr>
<td>Hippo</td>
<td>5</td>
</tr>
<tr>
<td>Cow</td>
<td>22</td>
</tr>
<tr>
<td>Giraffe</td>
<td>19</td>
</tr>
<tr>
<td>Sheep</td>
<td>19</td>
</tr>
<tr>
<td>Feral Hog</td>
<td>5</td>
</tr>
<tr>
<td>White Rhino</td>
<td>5</td>
</tr>
<tr>
<td>Horse</td>
<td>5</td>
</tr>
<tr>
<td>Harbor Seal</td>
<td>6</td>
</tr>
<tr>
<td>Dog</td>
<td>6</td>
</tr>
<tr>
<td>Flying Fox (Bat)</td>
<td>5</td>
</tr>
<tr>
<td>Short-tailed Shrew</td>
<td>7</td>
</tr>
<tr>
<td>Capybara</td>
<td>14</td>
</tr>
<tr>
<td>Gray Squirrel</td>
<td>11</td>
</tr>
<tr>
<td>Mouse</td>
<td>7</td>
</tr>
<tr>
<td>Rat</td>
<td>8</td>
</tr>
<tr>
<td>Lemming</td>
<td>6</td>
</tr>
<tr>
<td>Beaver</td>
<td>6</td>
</tr>
<tr>
<td>Rabbit</td>
<td>6</td>
</tr>
<tr>
<td>Gorilla</td>
<td>15</td>
</tr>
<tr>
<td>Chimpanzee</td>
<td>13</td>
</tr>
<tr>
<td>Human</td>
<td>15</td>
</tr>
<tr>
<td>Rhesus Macaque</td>
<td>15</td>
</tr>
<tr>
<td>Crab-eating Macaque</td>
<td>11</td>
</tr>
<tr>
<td>Two-toed Sloth</td>
<td>6</td>
</tr>
<tr>
<td>Elephant</td>
<td>19</td>
</tr>
</tbody>
</table>

Clades:
- Cetartiodactyla
  - Cetacea
  - Bovidae
  - Suidae
- Perissodactyla
- Carnivora
- Chiroptera
- Eulipotypha
- Rodentia
  - Caviomorpha
  - Sciuromorpha
  - Muroidea
  - Lagomorpha
- Primates
- Xenartha
- Proboscidea
- Afrotheria
Figure 3.3. Mammalian phylogenies suggest independent neurofilament medium (NF-M) lysine–serine–proline (KSP) repeat expansion events across several clades. (A) An established placental mammalian phylogeny was adapted (Murphy et al., 2001) to compare with the phylogeny generated from the NFM C-terminus alignment. Only species that were sequenced for NF-M exon 3 were indicated and species without sequencing were omitted for simplicity. Branching points for omitted species were collapsed as well for simplicity. Species that were sequenced for NF-M exon 3 but were not used to generate the phylogeny were incorporated at appropriate points based on other diverse mammalian phylogenies (Bininda-Emonds et al., 2007) and were indicated by dashed lines. (B) Molecular phylogenies of mammals were generated from the
ClustalW alignments of the entire NF-M C-terminal tail domain. Consensus tree construction and bootstrap analysis were performed utilizing the neighbor joining method with 10,000 trials and a random number generator seed of 111 (MegAlign, DNASTar, Inc.). Bootstrap values at tree nodes were calculated as the percentage that each particular node appeared during bootstrap analysis. Dashed lines represent nodes that collapsed in the strict consensus tree. NA = not available, meaning the bootstrap analysis failed to support the node. (A, B) Brackets and bars indicate higher-level taxonomic groups that were identified in the phylogenies. Mapping KSP repeat numbers onto the phylogenies reveals that KSPs expanded independently in several mammalian clades. The different colored branches indicate the KSP expansion events in both constructed trees.
low KSP repeat number while giraffe, cow, and sheep, members of *Bovidae*, diverged and their KSP repeats expanded to 19-22 (Fig. 3.3A and B). Similarly, feral hog, member of *Suidae*, diverged at an earlier point into another clade and its KSP repeats expanded to 19. This is supported by the differential patterning of the KSP repeats as feral hog expanded KXSPs and KXXSPs, while *Bovidae* members expanded almost exclusively KSPs (Table 3.2). Similar expansions appear to have occurred throughout the mammalian phylogeny, including in *Rodentia, Primates, and Proboscidea* (Elephant).

**Alignments of NF-M KSP Repeat Regions Reveals Conserved Patterning within Species Sub-groups**

Upon further inspection, the bimodal distributions appear to be sub-divided into three groups based upon their positions within the graphs (Fig. 3.2). Moreover, individual members of these sub-groups patterned their KSP repeats in a similar manner (Fig. 3.4). While the blue and green group species (Fig. 3.4A and B) appear to have patterned their KSP repeats similarly, the blue group uniquely patterned the first two KSP repeats within the repeat sub-domain (Fig. 3.4A). The red group contained the most divergence in KSP repeat patterning (Fig. 3.4C). An alignment of KSP repeat sub-domain of species from each of the three sub-groups, illustrated the distinguishing patterns of each group (Fig. 3.4D). It is interesting that the species within the red group appeared to have patterned their KSP repeats by adding repeats almost exclusively after the fourth consensus KSP repeat (Fig. 3.4D).
C  NF-M KSP repeat Sub-domain Alignment of Red KSP Patterning Group Species

D  NF-M KSP repeat Sub-domain Alignment of Species From Each KSP Patterning Group
Figure 3.4. Organization of lysine–serine–proline (KSP) repeats within the neurofilament medium (NF-M) C-terminal tail domain is conserved within, but not between, sub-groups of mammals. KSP repeat distribution, within the KSP repeat sub-domain, was determined by ClustalW aligning of protein sequences from mammals representing each of the groups identified through regression analysis. Majority = the amino acid sequence that is represented in more than 50% of individual sequences. A gap (−) in the sequence indicates an insertion event in less than 50% of the individual sequences. (A–B) Aligned proteins sequences from mammals within a group indicated that KSP repeat organization was conserved within a sub-group of mammals. KSP repeats were identified using colors originally assigned each sub-group. (C) Aligned proteins sequences from mammals within a group indicated that KSP repeat organization was conserved within a sub-group of mammals. KSP repeats were identified using colors originally assigned each sub-group (D) KSP repeat organization was not conserved between groups of mammals. Consensus KSP repeats were highlighted in gold.
Axonal Diameter in Mammals Correlates with KSP Repeat Number

The diameter distribution and numbers of axons were analyzed from motor axons of the 5th lumbar ventral roots of five mice, three rats, and two cows. All three species displayed typical bimodal distribution of axonal calibers (Fig. 3.5). The peaks in the distribution of the largest motor axons in mouse, rat, and cow were 6, 7, and 10 μm, respectively, suggesting motor axon caliber expansion in mammals (Fig. 3.5). Mouse and rat at six months of age have likely completed the majority of radial growth of motor axons (Garcia et al., 2003; Garcia et al., 2009; Rao et al., 2003; Rao et al., 2002; Rao et al., 1998; Zhu et al., 1997). However, seven month-old cows are still relatively immature and may not have completed axonal radial growth. Therefore, it is likely that the peak distribution of the largest group of axons in cow will be shifted to a greater caliber at completion of axonal radial growth. It should be emphasized that the axonal diameter data set is limited to three species and more experimentation is warranted.

Discussion:

NF-M KSP Repeats in Context with Mammalian Phylogeny

Evolutionary relationships of the structure of the NF-M C-terminus in mammals were examined to better understand its influence on axonal diameter. Results indicate that the C-terminus contains highly conserved Amino and Carboxy flanking sub-domains that surround a highly variable sub-domain with characteristic KSP repeats. The high variability of the KSP repeat sub-domain is
Figure 3.5. Expansion in the number of KSP repeats is correlated with larger axonal diameters. (A) Cross sections of the fifth lumbar motor (ventral) root of mouse, rat and cow. Scale bar, 10 μm. (B) Distribution of axonal diameters of fifth lumbar motor (ventral) root axons from mice, rats and cows. Mice and rats were aged six months, and cows were aged seven months. Each point represents the averaged distribution of axon diameters from the entire roots of five mice, three rats and two cows. The KSP repeat numbers of 7, 8 and 22 in mouse, rat and cow, respectively, positively correlate with the axonal diameter distributions.
due not only to higher substitution rates, but also due to 362 non-conserved insertion events of amino acid codons according to the total sequence alignment (data not shown). In contrast, the alignment contained few insertion events in the Amino and Carboxy sub-domains that were outside of the KSP repeat sub-domain (data not shown). The number of KSP repeats correspond directly to the length of the NF-M C-terminus, meaning that the expansion of KSP repeats is the predominant mechanism utilized for lengthening this domain amongst mammals.

Our initial pooled analysis revealed a general relationship between KSP expansion and head-body length (Fig. 3.2), suggesting a role for KSP expansion in axon diameter and therefore conduction velocity. However, a finer detailed phylogenetic analysis suggests that KSP expansion may have occurred independently across multiple clades as a convergent mechanism to influence axonal diameter. Mapping KSP repeat numbers onto mammalian phylogenies suggests that ancestral mammals contained relatively few KSP repeats (~5). Some present-day mammals maintain this number of KSP repeats. For example, we found no evidence of KSP expansion in the Carnivora, Chiroptera, and Perissodactyla (Fig 3.3A). But within several different clades, including Cetartiodactyla, Rodentia, Primates, and Proboscidea, independent KSP expansion events occurred that appear to have been adaptive as the expansions remain in present-day descendants. Moreover, the constructed trees (Fig. 3.3A and B) suggest that independent KSP expansion occurred in Rodentia at least three times as rat, gray squirrel, and capybara have 8, 11, and 14 KSP repeats,
respectively. We suggest that gray squirrel and capybara KSP expansion occurred independently, as gray squirrels developed an expansion of KXXSPs, while capybara expanded with KSPs. It is also possible that rat and capybara could be descended from a common ancestor as they both have increased numbers of KSP repeats. However, rat and capybara diverge from one another by several branch points in Rodentia (Fig. 3.3A and B), so the KSP expansions still may have occurred independently.

Independent expansion of KSP repeats occurring across several mammalian clades suggests that increasing the length of the C-terminus may be a common mechanism utilized to influence axonal diameter and may represent an example of convergent evolution. If expanding the length of NF-M C-terminus through the addition of KSP repeats is part of the mechanism utilized to determine axonal diameter, then, to our knowledge, this would represent the first functional enhancement associated with the expansion of a repetitive DNA element. It has become clear, though, that within individual species NF-M C-terminal expansion is not the only available mechanism for influencing axonal diameter. For example, despite having 22 KSP repeats, cows have a bimodal distribution of motor axons of the 5th lumbar root (Fig. 3.5B) with relatively small diameter axons, yet all axons contain the same NF-M protein. Therefore, expansion of NF-M C-terminus may only be part of the mechanism utilized to determine axonal diameter.
NF-M Mediated Radial Axonal Growth

We hypothesized that as mammals evolved larger body plans across different clades, selective pressures resulted in increased conduction velocities to maintain the rate of signal propagation. Furthermore, we proposed that expansion of the KSP repeats within the NF-M C-terminus might have been an evolutionary mechanism that resulted in increased axonal diameters. Direct measurements of nerve conduction velocity are extremely difficult to obtain from many of the species analyzed in this study. However, analysis of individual nerve fibers of adult cat hind-limb demonstrated a linear relationship of axonal diameter with conduction velocity, with the largest diameter fibers having the fastest conduction velocities (Hursh, 1939). Subsequent studies (Arbuthnott et al., 1980a; Arbuthnott et al., 1980b; Boyd and Kalu, 1979; Waxman, 1980; Westbury, 1982) as well as theoretical results (Rushton, 1951; Smith and Koles, 1970) support this finding. Moreover, direct measurements of nerve conduction velocities in human (Chang et al., 2006) and mouse (Garcia et al., 2003; Garcia et al., 2009) suggest that larger mammals have faster rates of conduction. Our results suggest that increased number of KSP repeats within NF-M is associated with larger axonal diameter (Fig 3.5). If the relationship between axonal diameter and conduction velocity holds for other mammals, then it is likely that an expanded NF-M C-terminus is correlated with faster conduction velocity for a subset of mammals.

It has long been thought that myelin-dependent phosphorylation of NF KSP repeats was required for radial axonal growth (de Waegh et al., 1992).
However, the generation of a mouse expressing full-length, KSP phosphorylation-incompetent NF-M has challenged the role of KSP phosphorylation in establishing axonal diameter (Garcia et al., 2009). These new results were not inconsistent with our proposal. It is possible that the overall length of NF-M C-terminus determines axonal diameter, as axonal diameter and conduction velocity were altered only in mice missing the entire NF-M C-terminus (Garcia et al., 2003). Recently, mathematical models of NF side arms suggest that human NF-M C-termini project the farthest from the NF core relative to NF-L and NF-H C-termini (Chang et al., 2009). The distance that NF-M side arms project appears to be largely dependent upon adjacent C-termini stoichiometry, amino acid sequence, and the charge of the residues (Chang et al., 2009). Since the amino acid compositions and overall charges of NF-M C-termini are similar across mammals (Table 3.3), it is reasonable to predict that lengthening the NF-M C-terminus would allow for it to project farther. Mechanistically, it has been proposed that NF C-terminal tails form cross-bridging structures with adjacent NFs or microtubules (Hirokawa et al., 1984; Hisanaga and Hirokawa, 1988). Alterations in the length of the KSP repeat sub-domain may have evolved as a means of extending the length of the cross-bridge, allowing for longer-range interactions that may provide greater structural stability in larger diameter axons. Our data suggests that increasing the overall length of the NF-M C-terminus by adding additional KSP repeats may be a potential mechanism for increasing axonal diameter.
NF-M KSP repeats may not be considered classic tandem repeats as they consist of tri- tetra- and pentapeptide repeats (KSP, KSD, KXSP, KXXSP) and are not typically adjacent to one another but interspersed along the sub-domain. Nevertheless, the number and patterning of KSP repeats was highly divergent between mammalian species (Table 3.2 and Fig. 3.4D). Moreover, the KSP repeat sub-domain is located within a region of NF-M that is required for radial growth (Garcia et al., 2003). We propose that much like tandem repeat variation affecting dog snout morphology (Fondon and Garner, 2004), changes in KSP repeat number within this highly divergent sub-domain of NF-M C-terminus appear to be important in altering axonal morphology and may have been a vital part of a mechanism for increasing conduction velocity to maintain the rate of signal propagation as subsets of larger mammals evolved.
CHAPTER 4

EXPANSION OF NF-M CARBOXY TERMINUS INCREASES AXONAL DIAMETER INDEPENDENT OF INCREASES IN CONDUCTION VELOCITY OR MYELIN THICKNESS

(This work was submitted for publication to a peer-reviewed journal.)

Devin M. Barry\textsuperscript{1,2}, William Stevenson\textsuperscript{3}, Brian G. Bober\textsuperscript{4}, Peter J. Wiese\textsuperscript{1}, Jeffrey M. Dale\textsuperscript{1,2}, Garet S. Barry\textsuperscript{1,2}, Nathan S. Byers\textsuperscript{1,2}, Jonathan D. Strope\textsuperscript{1,2}, Rakwoo Chang\textsuperscript{5}, David J. Schulz\textsuperscript{1}, Sameer Shah\textsuperscript{4}, Nigel A. Calcutt\textsuperscript{6}, Yeshitila Gebremichael\textsuperscript{3}, Michael L. Garcia\textsuperscript{1,2,*}

\textsuperscript{1}Department of Biological Sciences, \textsuperscript{2}C.S. Bond Life Sciences Center, University of Missouri, Columbia, MO 65211, \textsuperscript{3}Department of Biomedical Engineering, Wayne State University, Detroit, MI 48201, \textsuperscript{4}Department of Orthopaedic Surgery, University of California, San Diego, La Jolla, CA 92093, \textsuperscript{5}Department of Chemistry, Kwangwoon University, Seoul 139-701, Republic of Korea, and \textsuperscript{6}Department of Pathology, University of California San Diego, La Jolla, CA 92093.
Abstract:

Maturation of the peripheral nervous system requires specification of axonal diameter, which, in turn, has a significant influence on nerve conduction velocity. Radial axonal growth initiates with myelination, and is dependent upon the C-terminus region of neurofilament medium (NF-M). Molecular phylogenetic analysis in mammals suggested that expanded NF-M C-termini correlated with larger diameter axons. We utilized gene targeting and computational modeling to test this new hypothesis. Increasing the length of NF-M C-terminus in mice increased diameter of motor axons without altering neurofilament subunit stoichiometry. Computational modeling predicted that an expanded NF-M C-terminus extended farther from the neurofilament core independent of lysine-serine-proline (KSP) phosphorylation. However, expansion of NF-M C-terminus did not affect the distance between adjacent neurofilaments. Increased axonal diameter did not increase conduction velocity, possibly due to a failure to increase myelin thickness by the same proportion. Failure of myelin to compensate for larger axonal diameters suggested a lack of plasticity during the processes of myelination and radial axonal growth.

Keywords: neurofilament, axon diameter, radial growth, myelin thickness, nerve conduction velocity
Introduction:

Specification of axonal diameter is a key component of motor neuron function as it is a major axonal property that determines the velocity of signal conduction (Rushton, 1951; Waxman, 1980). Myelination of peripheral nerve fibers is necessary for rapid impulse transmission (Huxley and Stampfli, 1949) and occurs on axons that are >1µm (Duncan, 1934). Myelination increases conduction rates by preventing charge loss and reducing membrane capacitance (Huxley and Stampfli, 1949; Koles and Rasminsky, 1972; Rasminsky and Sears, 1972). Myelination also initiates cell biological changes in axons resulting in larger axonal diameters (de Waegh et al., 1992), which further decreases axonal resistance and contributes to increasing the rate of conduction (Boyd and Kalu, 1979; Hursh, 1939).

The large increase in axonal diameter that occurs with myelination is referred to as radial growth (Cleveland, 1996) and it is dependent upon both myelination (de Waegh et al., 1992) and neurofilaments (NFs) (Ohara et al., 1993; Zhu et al., 1997). NFs are obligate heteropolymers in vivo, and can be composed of neurofilament light (NF-L), neurofilament medium (NF-M), neurofilament heavy (NF-H) (Lee et al., 1993) and a-internexin (Yuan et al., 2006). The composition of NF heteropolymers varies with developmental stage (Shaw and Weber, 1982; Shen et al., 2010).

Gene targeting studies identified NF-M as the critical subunit that is required for radial growth (Elder et al., 1998a) and the NF-M C-terminus as the critical domain (Garcia et al., 2003). NF-M was originally thought to regulate axonal
diameter through myelin-dependent phosphorylation of lysine-serine-proline (KSP) repeats (de Waegh et al., 1992; Yin et al., 1998) located within the NF-M C-terminus (Levy et al., 1987; Myers et al., 1987). However, preventing NF-M KSP phosphorylation in NF-M<sup>S→A</sup> mice failed to recapitulate the reduction in radial growth of motor axons observed in NF-M C-terminally truncated mice (Garcia et al., 2003; Garcia et al., 2009). Taken together, these data suggest that NF-M C-terminus regulates axonal diameter by a mechanism that is independent of KSP phosphorylation.

To provide insight into the role of NF-M C-terminus in regulating axonal diameter, molecular phylogenetic analyses of NF-M exon 3, which codes for NF-M C-terminus, were performed across several clades of mammals (Barry et al., 2010). Sequence analysis suggested that the NF-M C-terminus was divided into three sub-domains, two conserved domains flanking a variable region. Sequencing also revealed that independent expansion events of NF-M C-terminus occurred within multiple clades of the consensus mammalian phylogenetic tree. All observed expansion events occurred by the addition of amino acids, including KSP repeats, within the variable sub-domain. Moreover, NF-M KSP repeat number and, consequently, NF-M C-terminal length correlated with axonal diameter in a subset of mammals. These data suggested that lengthening the NF-M C-terminus through expansion in the number of KSP repeats might have resulted in larger axonal diameters during mammalian evolution. To test this new hypothesis, we have now applied gene replacement and computational modeling techniques to increase the length of NF-M C-
terminus by replacing the endogenous NF-M C-terminus and its 7 KSP repeats
with the longer bovine C-terminus and its 22 KSP repeats.

**Experimental Procedures:**

**Generation of NF-M\textsuperscript{BovineTail} mice.**

All procedures were in compliance with the University of Missouri Animal
Care and Use Committee and with all local and federal laws governing the
humane treatment of animals. The murine nefm genomic clone was a generous
gift from J. P. Julien (Jacomy et al., 1999). The 7 kb NotI–BamHI fragment of
nefm genomic clone was digested with Accl to produce a ~2.2 kb fragment that
included mouse nefm Exon 3 as well as ~500 bp of 5' and 3' untranslated region
(UTR). Nefm Bovine exon 3 and its 3'UTR were PCR amplified from bovine
sperm DNA (provided as a gift by Jerry Taylor, University of Missouri) and cloned
into a T-Vector. The bovine exon 3 clone was then mutagenized to introduce a
BsrGI restriction site that is already present in mouse nefm exon 3. An Ndel site
was introduced in the mouse nefm 3'UTR by mutagenesis that was present in the
bovine nefm 3'UTR. The BsrGI-Ndel bovine fragment was cloned into the Accl-
Accl mouse fragment. To make the region of exon 3 5' to the BsrGI 100%
identical to bovine exon 3, amino acid codon 414 (NCBI Accession P08553)
within mouse exon 3 was mutagenized from a serine codon to an alanine codon.
The Accl-Accl fragment with bovine exon 3 was then cloned back into the 7 kb
NotI–BamHI fragment. Nefm 5'UTR and 3'UTR regions were amplified from
mouse 129SvEv Genomic DNA to increase homology arm lengths from 5kb to a
total of 8kb. Diphtheria toxin α (DTA) was introduced at the 3’end of the construct as a negative selection marker. Finally, the nefl 3’UTR and a PGK Neo cassette were cloned at the 3’ end of bovine exon 3 (inserted at the SalI and MluI sites which were cloned into the nefm 3’UTR using a linker). The targeting construct was linearized with AatII and electroporated into mouse 129SvEv ES cells (Millipore Corporation, Billerica, MA, US) by the MU Transgenic Core and selected with G418 at 250µg/ml (Joyner, 1994). Drug-resistant colonies were amplified and screened by PCR at the 3’ end and Southern blot at the 5’ end. DNA sequence analysis (MU DNA Core) was used to ensure incorporation of bovine nefm exon 3. Two out of 192 ES clones were identified to have undergone homologous recombination at both the 5’ and 3’ ends of the gene to produce the NF-M\textsuperscript{BovineTail} targeted allele. One of the positive ES cell clones was injected into mouse C57Bl6 blastocysts and the MU Transgenic Core implanted blastocysts into the uteri of pseudo-pregnant surrogates. Four chimeric male mice were identified from the surrogates. Germ line transmission of the NF-M\textsuperscript{BovineTail} allele was confirmed by PCR amplification of genomic DNA purified from tail biopsies using Ex Taq™ (Clontech Laboratories, Inc., Madison, WI, US) with the following primers: Forward Primer-5’AAACTCCTAGAGGGGAAGAGACCAGAT3’, Mouse Reverse Primer-5’CAACTCCTCTGCGATGGCTGTGA3’, Bovine Reverse Primer-5’CTCCTTTTCTCCTTCTTCTTATA3’. Mice were bred to homozygosity (NF-M\textsuperscript{BovineTail}) and wild type littermates were used as controls for the analyses.
Detection and quantification of neurofilament and tubulin proteins by immunoblotting.

Sciatic nerve and spinal cord tissues were dissected and homogenized on ice in a buffer containing 50 mM Tris, pH 7.5, 0.5 mM EDTA, pH 8, and protease inhibitors were added according to manufacturer's protocol (Complete Mini, Roche, Mannheim, Germany). An equal volume of a solution containing 50 mM Tris, pH 7.5, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 2% SDS was added, and the homogenates were sonicated for 20 s, boiled for 10 min, and clarified by centrifugation at 16,000 g for 10 min. Protein concentration was determined using the Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA, US). Protein extracts were separated on 7.5% SDS-polyacrylamide gels and transferred onto nitrocellulose membrane or stained with Coomassie-blue. Mouse monoclonal antibodies to NF-L (MCA-DA2, EnCor Biotechnology, Gainesville, FL, US) and NF-M (RMO44, Abcam, Cambridge, MA, US), were used to identify each protein. NF-H was identified with a chicken polyclonal antibody that recognizes mammalian subunits (CPCA-NF-H, EnCor Biotechnology). NF-H and NF-M were detected using a mouse monoclonal antibody that recognizes the proteins in a phospho-dependent manner (SMI-31, Covance, Emeryville, CA, US). Neuron specific, βIII-tubulin was identified with a rabbit monoclonal antibody (TUJI, Covance, Emeryville, CA, US). Mouse chicken, and rabbit primary antibodies were detected with donkey anti-mouse, goat anti-chicken, and donkey anti-rabbit secondary antibodies conjugated to IRdye-700X® infrared fluorophores (Rockland, Gilbertsville, PA, US), respectively.
Immunoreactive bands were visualized by infrared detection with an Odyssey image scanner (LICOR Biosciences, Lincoln, NE, US).

Absolute intensities of immunoreactive bands were obtained using Photoshop (Adobe Systems Inc., San Jose, CA, US). Relative optical densities (RODs) of immunoreactive bands were calculated as follows: 

\[
\frac{[\text{SMI-31 mean intensity} - \text{background mean intensity}] \times \text{number of pixels}}{[(\text{CPCA-NF-H mean intensity} - \text{background mean intensity}) \times \text{number of pixels}]}.
\]

Average SMI-31 RODs were analyzed for statistical significance by one-way ANOVA with Holm-Sidak post hoc analysis for pair-wise comparisons (SigmaPlot, Systat Software, Inc.)

**Tissue preparation, axon morphological analysis, EM and NF nearest-neighbor analysis**

Mice were sacrificed and perfused intracardially with 2.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M Sorenson’s phosphate buffer, pH 7.2, and post-fixed overnight in the same buffer. Fifth lumbar nerve roots were dissected, treated with 2% osmium tetroxide, washed, dehydrated, and embedded in Epon-Araldite resin. Thick sections (0.75 µm) for light microscopy were stained with p-phenylenediamine. Images of transverse sections of L5 motor and sensory axons were collected with a Zeiss Axio Imager A1 light microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany). Transverse sections of L5 motor and sensory axons were analyzed in at least five mice per genotype and age group. Entire roots were imaged, imaging thresholds were selected individually, and the
cross-sectional area of each axon was calculated and reported as a diameter of
a circle of equivalent area using the AxioVision Digital Image Processing
Software (Carl Zeiss MicroImaging). Axon diameters were grouped into 0.5µm
bins. G-ratios were estimated by measuring the axonal diameter and fiber
diameter of individual axons for 10% of all axons per motor root using AxioVision
Digital Image Processing Software (Carl Zeiss MicroImaging). The total number
of axons was analyzed for statistical significance using a two way ANOVA
(SigmaPlot, Systat Software, Inc.) Bimodal distributions of motor axon diameter
were analyzed for overall statistical significance using Mann–Whitney U test. G-
ratios were analyzed for statistical significance by three-way ANOVA with Holm-
Sidak post hoc analysis for pair-wise comparisons.

Thin sections (60–90 nm) were cut from prepared resin blocks with a Leica
Ultracut E ultramicrotome, stained with 1% aqueous uranyl acetate for 15 min.
followed by bismuth subnitrate for 2 min. Images of selected neurons were
collected at 80 kV with a JEOL 1400 Transmission Electron Microscope at
magnifications of 5000, 10,000, or 20,000. Microtubules were counted within
axoplasm. Neurofilaments were traced and nearest-neighbor calculations made.
Raw NF spacing numbers were acquired using IMOD software (University of
Colorado, Boulder, CO, US), and NF NND data were grouped into 4 nm bins
using NCMIR Pointzilla (University of California, San Diego, CA, US). NF
numbers were analyzed for statistical significance using Student’s t-test (small
motor and sensory axons) or Mann-Whitney U test (large motor axons)
(SigmaPlot, Systat Software, Inc.)
**Neurofilament clustering.**

Average neurofilament spacing was determined as described previously (Garcia et al., 2003, 2009), by distributing identified neurofilaments in uniform arrays across the effective cross-sectional area of an axon. Briefly, cross-sectional area was estimated by tracing axoplasmic regions of the same digitized electron micrographs used to identify neurofilaments. Neurofilaments were organized in concentric hexagonal “rings” of equilateral triangles, with average neurofilament spacing calculated as the side length of one triangle. Neurofilament clustering was defined as the ratio of average filament spacing to nearest-neighbor filament spacing, with higher ratios implying more clustered (less uniformly distributed) neurofilaments. Analysis was performed using MATLAB 6.5 (The MathWorks). Clustering, NF density, microtubule density and microtubule/ NF ratios were analyzed for statistical significance by two-way ANOVA (SigmaPlot, Systat Software, Inc.)

**Nerve conduction velocity measurements.**

Nerve conduction velocities were measured in the sciatic nerve, interosseus muscle system of 6 month old mice (Calcutt et al., 1990). In brief, mice were anesthetized with halothane (4% in O₂ for induction, 2–3% for maintenance), and rectal temperature was maintained at 37°C by a heating lamp and thermal pad connected to a temperature regulator and the rectal thermistor probe. The sciatic nerve was stimulated with single supramaximal square wave pulses (4–8 V and
0.05 ms duration) via fine needle electrodes placed at the sciatic notch and Achilles tendon. Evoked electromyograms were recorded from the interosseus muscles of the ipsilateral foot via two fine needle electrodes and displayed on a digital storage oscilloscope. The distance between the two sites of stimulation was measured using calipers, and conduction velocity was calculated as previously described (Calcutt et al., 1990). Measurements were made in triplicate from a minimum of seven animals per genotype, and the median was used as the measure of velocity. Values were compared for overall statistical significance by Student’s t-test (InStat, GraphPad Software, La Jolla, CA, US).

**Computational simulations of NF C-termini.**

The computational part of the present study employs the sequence-based, coarse-grained NF brush model of Chang et al. (Chang et al., 2009). The model consists of long flexible side arms tethered to a central rigid cylindrical core representing the 10-nm-diameter NF backbone (Figure 1) (Chang et al., 2009). In this model, NF C-termini were modeled at amino acid resolution, with each amino acid represented by a sphere of diameter $\sigma_s = 0.6$ nm along with the corresponding charge. Charges consistent with a neutral pH were assigned to all ionizable residues as well as all serine residues of the KSP repeat motifs in accordance to the phosphorylation state of the filament. Counterions were added explicitly to achieve electroneutrality, whereas solvent was treated implicitly through the use of appropriate dielectric constant (or Bjerrum length $l_B = 0.7$ nm for water solution at $T = 300$K). The interaction potential between different
objects was treated as the sum of hard sphere (or rod) and electrostatic interactions. The model is based on the sequence and stoichiometry of mouse neurofilament (Stevenson et al., 2011). A model of mouse-bovine chimera (NF-M\textsuperscript{BovineTail}) was generated by substituting the NF-M sequence of the mouse with that of the cow. The sequences of mouse neurofilament light, medium and heavy side arm polypeptides were retrieved from the Uniprot Consortium with accession numbers P08551, P08553, and P19246 (Jain et al., 2009). The sequence of bovine NF-M was retrieved from NCBI with accession number O77788 (Benson et al., 2009; Trimpin et al., 2004). The NF system was placed in a simulation box 400 x 400 wide and 50 nm deep. Periodic boundary condition was imposed along the longitudinal axis of the filament, allowing a representation of an infinitely long filament. Then, off-lattice canonical ensemble Monte Carlo (MC) simulations were conducted under phosphorylated, dephosphorylated and NF-M phospho-incompetent conditions. NF-M phospho-incompetence was attained from phosphorylated NF system by replacing all the serine in the NF-M KSP repeats by alanine, thus defeating the negative charge change associated with phosphorylation. The MC simulations were carried out under 150 mM ionic conditions and was accomplished by employing the screened coulomb (Debye-Hückel) potential (McQuarrie, 2000). More details on the model and simulation procedures can be found in Stevenson et al. (Stevenson et al., 2011). Averages of radius of gyration for each NF subunit in each condition were analyzed by Student’s $t$-test.
Mathematical modeling of nerve conduction velocity.

To estimate the effect of a change in membrane capacitance (as reflected by the change in g-ratio between wild type and NF-M^{BovineTail} mice) on conduction velocity, a mathematical model of the axon fashioned after linear cable theory (Cotterill, 2002) was employed to determine the velocity of action potential propagation down multiple nodes in a cable as follows:

\[ V(X,T) = \frac{V_0}{2}(e^{-X} \cdot \text{erfc}(X/2\sqrt{T}) - \sqrt{T}) + e^{X} \cdot \text{erfc}(X/2\sqrt{T} + \sqrt{T}) + V_{\text{rest}} \]

where \( X \) represents the electrotonic length \( x/\lambda \) and \( T \) represents \( t/\tau_m \). This allowed changes in resistance and capacitance to be introduced into the model as \( \lambda = \sqrt{(r_m/r_i)} = \sqrt{((R_m*d)/(R_i*4))) \) and \( \tau_m = R_m \cdot C_m \). Values of key properties measured directly from sciatic nerve axons as reported in the literature (Kriz et al., 2000) were incorporated as follows: \( V_0 \) (action potential amplitude) = 87.7 mV, \( V_{\text{rest}} = -75.5 \) mV, \( R_m = 25.8 \) MΩ cm\(^2\). Other variables were determined based on similar measurements taken from other sources, including \( R_i = 200 \) Ω cm and \( C_m = 0.6 \) µF cm\(^{-2}\) (Squire, 2003) and threshold voltage of -55 mV. Time to threshold values were obtained from the nominal model (wild type) with \( X \) representing the internodal segment electrotonic length, and then velocity was simply calculated as distance per unit time. Finally, this velocity was scaled to match that of the biological data for wild type mice (i.e. 47.3 m/s) to estimate total distance over which the recordings were made. Factors of diameter and capacitance were altered to mimic changes observed in the experimental data. Specifically, conduction velocity of an expansion solely in axon diameter with all other factors remaining constant was estimated to predict conduction velocity for the axon.
diameter shift seen in the NF-M\textsuperscript{BovineTail} mice. A change in $C_m$ was estimated based on values of g-ratio proportionally as follows: $C_m \propto \frac{1}{(D-d)^2} = \frac{k}{(D-d)^2}$

where $D$ represents fiber diameter and $d$ represents axon diameter. The $k$ value was solved based on the nominal model and then G-ratio (fiber and axon diameter) values were substituted from the NF-M\textsuperscript{BovineTail} mice to determine the change in capacitance relative to the nominal model. This was then incorporated into a final model that included the increase in axon diameter as well as the predicted change in capacitance.

**Results:**

**Generation of chimeric NF-M gene through gene replacement of murine nefm exon 3 with bovine exon 3.**

To replace the endogenous nefm exon 3 with bovine exon 3, a segment of murine nefm containing exon 3 (~2.2 kb AccI/Accl fragment) was subcloned into pBluescript. Bovine exon 3 was PCR amplified (data not shown) from bovine sperm DNA, and subcloned into the murine ~2.2kb AccI/Accl nefm fragment using BsrGI and Ndel sites. The modified AccI/Accl nefm with bovine exon 3 fragment was cloned back into endogenous nefm. Replacing murine nefm exon 3 with bovine exon 3 generated a chimeric gene in which exon 1 and 2 were derived from the endogenous murine nefm while exon 3 was derived entirely from bovine nefm (Fig 4.1A). Replacing endogenous exon 3 with bovine exon 3 resulted in a tail domain that was 76 amino acids longer and contained 15
Figure 4.1. Generation of an NF-M<sup>BovineTail</sup> chimeric protein in mice by replacement of murine nefm exon 3 with bovine exon 3. Construction of an NF-M<sup>BovineTail</sup> allele in which murine exon 3 was replaced with bovine exon 3. Left, the three exons of murine nefm were identified by the white boxes interrupted by two introns. Bovine exon 3 was identified by the dark gray box marked ‘Bovine.’ ATG identified the translation initiation codon. Dashed lines indicated the two regions where homologous recombination could take place between the targeting vector and the endogenous nefm allele (A). Bovine NF-M C-terminus. The conserved sequence “klegee” (in bold) marked the end of the rod domain for all three neurofilament subunits. All KSP, KXSP, and KXXSP motifs were highlighted in bold uppercase letters, as well as the variant KSD (B). NF-M NCBI accession number O77788. Mouse genomic DNA was screened for targeting of the bovine nefm exon 3 using three primers for PCR based genotyping (C). Left, schematic representation of endogenous mouse nefm exon 3 and bovine nefm exon 3 with the approximate annealing sites of PCR primers and predicted sizes of amplified regions. Right, genotyping the nefm loci by PCR amplification of genomic DNA isolated from mouse-tail biopsies. Targeted and wild type PCR products were identified with arrows.
additional KSP repeats (Fig. 4.1B). The final targeting construct contained 8 kb of homology, including an added 3’ untranslated region (3’UTR) and polyadenylation signal from the murine nefl gene and neomycin phosphotransferase gene (Fig. 4.1A). Following ES cell electroporation, two out of 192 clones were identified to have recombined the modified nefm construct into one of the endogenous nefm alleles. The clones were injected into C57BL/6 blastocysts to produce chimeric animals that when bred to C57BL/6 mice transmitted the chimeric nefm (referred to as NF-M^{BovineTail}) allele to their progeny. Mating pairs of mice heterozygous for the NF-M^{BovineTail} allele produced homozygous animals (Fig. 4.1C) at the expected Mendelian frequency. The homozygous progeny were viable, fertile and displayed no overt phenotype through 2 years, the oldest age analyzed.

**Expression of NF-M^{BovineTail} decreased electrophoretic mobility of NF-M.**

Upon SDS-PAGE fractionation, mouse NF-M subunits in sciatic nerve extracts taken from 2 and 6-month-old mice migrated at a molecular weight of ~140 kiloDaltons (kDa) (Fig. 4.2A and 4.2D). Coomassie blue staining revealed that NF-M^{BovineTail} migrated at ~160 kDa (Fig. 4.2A and Fig 4.2D). Immunoblotting with an antibody specific to an epitope at the end of the NF-M rod domain (RM044, Abcam) confirmed the difference in the electrophoretic mobilities of the wild type and NF-M^{BovineTail} proteins and confirmed similar expression levels at 2 (Fig. 4.2B) and 6 (Fig. 4.2E) months. Moreover, similar levels of NF-L (MCA-DA2, EnCor Biotechnology), NF-H (CPCA-NF-H, EnCor Biotechnology), and the
Figure 4.2. Expression of NF-M\textsuperscript{BovineTail} had no effect on relative stoichiometries and accumulated levels of NF-L, NF-M, and NF-H but resulted in decreased NF-H phosphorylation. Parallel immunoblots of 2 and 6 month sciatic nerve extracts from wild type, NF-M\textsuperscript{BovineTail/wt}, and NF-M\textsuperscript{BovineTail/BovineTail} mice were fractionated on 7.5% SDS polyacrylamide gels and stained with Coomassie blue (A and D) or immunoblotted (B and E) with antibodies that recognize NF-H in a phospho-dependent (SMI31) and independent (CPCA-NF-H) manner, NF-M (RMO-44), NF-L (DA-2) and the neuron-specific βIII-tubulin (TUJI) (E, 6 month). The apparent molecular weight of the chimeric NF-M\textsuperscript{BovineTail} protein was increased (asterisk). NF subunit stoichiometry and βIII-tubulin in sciatic nerve were not affected by the expression of NF-M\textsuperscript{BovineTail}. SMI-31 immunoblotting revealed an apparent decrease of NF-H phosphorylation in NF-M\textsuperscript{BovineTail} mice relative to wild type. Relative optical density of SMI-31 (C) indicated a significant decrease of NF-H phosphorylation in NF-M\textsuperscript{BovineTail} heterozygous and homozygous mice relative to wild type. Average SMI-31 relative optical densities were analyzed for statistical significance by one-way ANOVA followed by Holm-Sidak post-hoc analysis for pair-wise comparisons. *, $p < 0.05$. Error bars = SEM. N= 3.
neuron-specific βIII-tubulin isoform (TUJI, Covance) were also observed in wild type and NF-M<sup>BovineTail</sup> extracts at 2 (Fig 4.2B, except βIII-tubulin) and 6 (Fig. 4.2E) months, demonstrating that NF-M<sup>BovineTail</sup> did not affect accumulation of the other major axonal cytoskeletal proteins in sciatic nerve. Detection of phospho-epitopes of NF-H recognized by SMI-31 (SMI-31, Covance) revealed an apparent decrease in NF-H phosphorylation in NF-M<sup>BovineTail</sup> mice at 2 (Fig. 4.2B) and 6 (Fig. 4.2D) months. Relative optical density (ROD) of SMI-31 immunoblots revealed a decrease in NF-H phosphorylation of NF-M<sup>BovineTail</sup> heterozygous and homozygous mice relative to wild type (Fig. 4.2C). Statistical analysis of SMI-31 ROD by one-way ANOVA followed with Holm-Sidak post hoc analysis for pairwise comparisons indicated a significant difference between wild type, NF-M<sup>BovineTail</sup> heterozygous and homozygous mice. However, this compensatory decrease in phosphorylation of NF-H was unlikely to affect radial growth as preventing all NF-H KSP phosphorylation through the deletion of NF-H C-terminus did not affect radial growth (Garcia et al., 2003; Rao et al., 2002).

**Increasing the length of the NF-M C-terminus increased radial growth of large motor axons.**

To determine whether lengthening the NF-M C-terminus influences radial axonal growth and survival, the size of all axons within the 5<sup>th</sup> lumbar motor root was determined at 2 and 6 months (Fig. 4.3A). Axons were counted (Fig. 4.3B) and cross-sectional areas were measured for all axons at both time points and
Figure 4.3. Increasing the length of the NF-M C-terminus resulted in increased radial growth of large motor axons. Transverse sections of L5 motor (ventral) root axons from wild type and NF-M\textsuperscript{BovineTail} homozygous mice (A). Scale bar = 10µm. Number of axons in L5 motor roots from 2 and 6-month-old wild type and NF-M\textsuperscript{BovineTail} mice (B). Counts are average from five animals for each genotype. Distribution of axonal diameters in motor axons of 2 (C) or 6 (D) month-old wild type and NF-M\textsuperscript{BovineTail} mice. Peak axonal diameter was initially reduced in small motor axons (C). However, by 6 months, peak diameters were indistinguishable from wild type (D). Peak axonal diameter was increased only in large motor axons at both 2 and 6 months. Points represent the averaged distribution of axon diameters from the entire roots of five mice for each genotype and age group. Axonal diameter distributions at each time point were analyzed for overall statistical significance using Mann-Whitney $U$ test. There was a statistically significant difference between diameter distributions of wild type versus NF-M\textsuperscript{BovineTail} mice ($p< 0.001$). N= 5.
corresponding diameters were calculated (Fig. 4.3C and D). Accumulation of wild type and NF-M\textsuperscript{BovineTail} protein yielded bimodal distributions of motor axons (Fig. 4.3C and D). At 2 and 6 months, the peak diameter of large motor axons from NF-M\textsuperscript{BovineTail} mice was increased relative to wild type littermates (Fig. 4.3C and D). Peak diameters in large NF-M\textsuperscript{BovineTail} axons were 1 µm greater than large wild type axons at 2 months and 0.5µm greater than large wild type axons at 6 months. Statistical analysis by Mann-Whitney U tests indicated a significant difference in the diameter distribution of motor axons at both 2 and 6 months. Interestingly, at 2 months the peak diameter of small motor axons from NF-M\textsuperscript{BovineTail} mice was 0.5 µm smaller. However, there was no difference in peak diameters for small motor axons at 6 months. The total number of motor axons tended to be lower in NF-M\textsuperscript{BovineTail} mice (Fig. 4.3B) but the difference was not statistically significant. These results demonstrate that extending the length of NF-M C-terminus expanded axonal diameter of large motor axons.

**Computational modeling of NF C-termini predicted that NF-M\textsuperscript{BovineTail} C-terminus extended farther from the NF core than wild type.**

Phylogenetic analysis suggested that longer NF-M C-termini correlated with larger axonal diameters in a subset of mammals (Barry et al., 2010). Moreover, computational modeling of human NF-L, NF-M, and NF-H C-termini under salt free condition suggested that the NF-M C-terminus extended the farthest from the NF core (Chang et al., 2009). Computational modeling was therefore utilized to determine if increasing the length of NF-M C-terminus increased the lateral
extension of NF-M C-terminus from the filament core and if increased lateral extension was dependent upon KSP phosphorylation. The structural organization of all NF C-termini was analyzed under the following conditions for wild type murine NF-M and murine NF-M in which the C-terminus was replaced by bovine C-terminus (NF-M<sub>BovineTail</sub>): (1) dephosphorylated KSP repeats, (2) KSP phospho-incompetent (S→A) and (3) phosphorylated KSP repeats. Simulations were performed in 150 mM monovalent salt conditions (Fig. 4.4A and B). To examine the effects of charge distribution and stoichiometry on lateral extension of wild type and NF-M<sup>BovineTail</sup> C-termini, the structural organization of NF heteropolymers was visually inspected with representative snapshots taken from Monte Carlo (MC) simulations (Fig. 4.4A and B). Qualitatively, NF-M C-terminus from NF-M<sup>BovineTail</sup> axons extended farther from the NF core independent of KSP phosphorylation (Fig. 4.4A and B).

Radius of gyration has been previously utilized to quantify the lateral extension of each NF C-terminus from the NF core (Chang et al., 2009; Stevenson et al., 2011). Radius of gyration for each NF C-terminus under 150 mM monovalent salt conditions was determined by sampling data every 1000 iterations of the MC simulation steps (from eight independent simulations) so that a total of 8000 equilibrated configurations could be selected for averaging. The phosphorylation-mediated changes in the NF C-termini were then quantified using the \( \langle Rg \rangle \) values determined from this analysis. In particular, the radial (perpendicular to the core of the filament) component \( \langle Rg \rangle_{\perp} \) that best describes the lateral extension of NF C-terminus was calculated. Replacing murine NF-M C-
Figure 4.4. Expansion of NF-M C-terminus resulted in increased extension from the core of the filament. Representative snapshots of all NF C-termini with phosphorylated wild type NF-M (A) and phosphorylated NF-M\textsuperscript{BovineTail} (B) in 150 mM monovalent salt (A and B) solution. Green side arms= NF-L C-terminus; blue side arms= NF-M C-terminus; red side arms= NF-H C-terminus. The average distance each NF C-termini extended from the filament core, referred to as radius of gyration, was determined in dephosphorylated (C), phospho-incompetent (NF-M\textsuperscript{S->A}) (D) and phosphorylated (E) states in 150mM monovalent salt (C, D and E) solution. In 150mM salt, NF-M\textsuperscript{BovineTail} C-terminus extended farther from the filament core independent of C-terminal KSP phosphorylation. Averages of radius of gyration for each NF subunit in each condition were analyzed by Student’s t-test. **, p< 0.001. Error bars are SEM. N= 8. Average monomer density profiles of NF-M C-termini in 150mM monovalent salt solution (F). Arrows indicated cutoff value for maximum lateral extension of each NF-M C-termini from wild type and NF-M\textsuperscript{BovineTail}. N= 8.
terminus with bovine C-terminus had no affect on NF-L and NF-H extension (Fig. 4.4C). Interestingly, NF-M\textsuperscript{BovineTail} C-terminus extended farther from the NF core than wild type NF-M independent of NF-M KSP phosphorylation (Fig. 4.4C-E). Statistical analysis by Student’s \( t \) test indicated significant differences in radius of gyration between NF-M C-termini of wild type and NF-M\textsuperscript{BovineTail} in each condition.

Ensemble-averaged monomer density profile \( \langle \rho(r) \rangle \) was also used to estimate extension of NF C-termini from the NF core. Unlike radius of gyration, monomer density profiles of NF C-termini estimated the maximum lateral extension of NF C-termini above a cutoff value (Chang et al., 2009; Stevenson et al., 2011). This quantity measured the probability of finding monomers as a function of the radial distance \( r \) from the filament core. For each NF side arm, \( \rho(r) \) was calculated by counting the number of monomers or CG sites that were within concentric cylinders bounded by the radii \( r \) and \( r + \delta r \). For any one particular configuration, the density profile \( \rho(r) \) of a particular type of C-terminus was obtained by summing over all C-termini belonging to the same type and then normalizing by the total number of C-termini of that type. This was then averaged over 8000 configurations obtained from eight independent simulations. While \( \langle \rho(r) \rangle \) served as a complementary measure for describing the C-terminal brush structure quantitatively, it also provided valuable information on the maximum lateral extension of individual C-termini. By noting the cutoff radius \( r_c \) at which \( \langle \rho(r) \rangle \) vanished, one can estimate and compare the range of the maximum lateral extensions of NF C-termini. Under 150mM monovalent salt conditions, the maximum lateral extension of phosphorylated NF-M C-termini in NF-M\textsuperscript{BovineTail}
was predicted to be farther than wild type (Fig. 4.4F).

**Expression of NF-M\textsuperscript{BovineTail} did not affect neurofilament spacing or clustering in small and large motor axons.**

To determine whether increasing the length of the NF-M C-terminus altered NF spacing, number and organization within peripheral myelinated axons, 5\textsuperscript{th} lumbar sensory, small motor and large motor axon cross sections from wild type and NF-M\textsuperscript{BovineTail} were visualized by electron microscopy. NFs were analyzed from axons that were at or near the peak diameter for each class of axon analyzed. NF spacing and number were quantified by calculating nearest neighbor distances (NND) of adjacent NFs within axoplasm of small motor axons at 2 (Fig. 4.5A and 4.5B) and 6 (Fig. 4.5C and 4.5D) months, as well as large motor axons at 2 (Fig. 4.5E and 6F) and 6 (Fig. 4.5G and H) months. The peak in NF spacing was unaffected in NF-M\textsuperscript{BovineTail} small motor (Fig. 4.5A and C) and large motor (Fig. 4.5E and G) axons when compared to wild type. Peak distances of NFs in wild type and NF-M\textsuperscript{BovineTail} small and large motor axoplasm were identical at 2 and 6 months. NF number in small motor axons was unaffected at 2 months (Fig. 4.5B). There were slightly fewer NFs in NF-M\textsuperscript{BovineTail} large motor axons at 2 months (Fig. 4.5F) and in small and large motor axons at 6 months (Fig. 4.5D and 4.5H) relative to axons from age-matched wild type mice but the differences were not statistically significant.
Figure 4.5. Neurofilament spacing was unaffected in motor axons of NF-M\textsuperscript{BovineTail} mice. Distribution of nearest-neighbor distances (NND) was determined for small motor (A and C) and large motor (E and G) axons from wild type and NF-M\textsuperscript{BovineTail} mice at 2 (A and E) and 6 (C and G) months. Additionally, NF numbers were determined for small motor (B and D) and large motor (F and H) axons from wild type and NF-M\textsuperscript{BovineTail} mice at 2 (B and F) and 6 (D and H) months. Peak NND was unaffected for small and large motor axons of NF-M\textsuperscript{BovineTail} mice at either time point. Moreover, NF number in small and large motor axons was not significantly affected in NF-M\textsuperscript{BovineTail} mice at either time point. Points represent averaged frequencies of NF nearest neighbor distances in axons from five mice per genotype and age group. Average number of neurofilaments was analyzed for statistical significance by Student's t-test. Error bars = SEM. N= 5.
Neurofilament clustering was measured by identifying the positions of all NFs in small and large motor axons and calculating the ratio of average filament spacing to nearest-neighbor distance. Higher ratios implied less uniformly distributed NFs. NFs within an axon were redistributed into concentric hexagonal rings of equilateral triangles over a cross sectional area equal to the cross sectional area of the axon. Average NF spacing was then defined as the edge length of each triangle. A perfectly ordered array of NFs would have, therefore, yielded a value of 1.0. As expected, the NF array in wild-type mice diverged from a perfectly ordered arrangement (Fig. 4.6A and 4.6B) at both 2 and 6 months. In large motor axons, NF clustering indexes were slightly less in NF-M\textsuperscript{BovineTail} at 2 and 6 months (Fig. 4.6B) relative to wild type suggesting a more ordered array of NFs. However, statistical analysis by two-way ANOVA indicated the difference in NF clustering between wild type and NF-M\textsuperscript{BovineTail} was not significant. NF clustering indexes of small motor axons were slightly less at 2 months but greater at 6 months when compared to wild type mice (Fig. 4.6A) but the differences were not statistically significant. NF densities (number of NFs/ µm\(^2\) of axoplasm) small motor (Fig. 4.6C) and large motor (Fig. 4.6D) axons were not significantly different between wild type and NF-M\textsuperscript{BovineTail} at 2 and 6 months.

**Motor nerve conduction velocity was unaffected in NF-M\textsuperscript{BovineTail} mice.**

Axonal diameter was a primary determinant of conduction velocity in myelinated axons (Boyd and Kalu, 1979; Garcia et al., 2003; Hursh, 1939). Therefore, motor nerve conduction velocities (MNCVs) were measured in the
Figure 4.6. Neurofilament clustering and density were unaffected in NF-motor axons of NF-M\textsuperscript{BovineTail} mice. Neurofilament clustering, defined as the ratio of average filament spacing to nearest-neighbor spacing, was calculated in small motor (A) and large motor (B) axons of wild type and NF-M\textsuperscript{BovineTail} mice at 2 and 6 months. Clustering was unaffected in small and large motor axons in NF-M\textsuperscript{BovineTail} mice at 2 and 6 months. Neurofilament densities (number of NFs/ \( \mu m^2 \) of axoplasm) were calculated from small motor (C) and large motor (D) axons of wild type and NF-M\textsuperscript{BovineTail} mice at 2 and 6 months. Densities tended to be reduced in small motor axons at 6 months and reduced in large motor axons at 2 months in NF-M\textsuperscript{BovineTail} mice. However, the differences were not statistically significant. Averages were analyzed for statistical significance by two-way ANOVA. Error bars = SEM. N= 5.
sciatic nerve of 6-month-old wild type and NF-M\textsuperscript{BovineTail} mice (7 animals per genotype). MNCV was indistinguishable in NF-M\textsuperscript{BovineTail} relative to wild type littermates (Fig. 4.7A).

**Myelin expansion was attenuated in NF-M\textsuperscript{BovineTail} motor axons.**

To determine whether myelination was altered in NF-M\textsuperscript{BovineTail} motor and sensory axons, myelin thickness and g-ratios were calculated by measuring axon diameter and fiber (myelin and axon) diameter for 10% of randomly sampled axons from the 5\textsuperscript{th} lumbar motor roots (Fig. 4.7B). At both 2 and 6 months myelin thickness was reduced for small motor axons of NF-M\textsuperscript{BovineTail} mice relative to wild type (Table 1). Myelin thickness of large (> 4\textmu m) motor axons from NF-M\textsuperscript{BovineTail} mice was similar to that of wild type mice at 2 months but by 6 months was significantly lower, reflecting a failure to increase at the same rate as myelin of wild type mice (Table 1). Analysis of myelin thickness by three-way ANOVA followed with Holm-Sidak post hoc analysis for pair-wise comparisons indicated a significant difference in myelin thickness between wild type and NF-M\textsuperscript{BovineTail} mice for small motor axons at 2 and 6 months and for large motor axons at 6 months. At both 2 and 6 months (Table 1), both g-ratios of small and large motor axons in NF-M\textsuperscript{BovineTail} mice were significantly increased, indicating that the relative thickness of myelin was reduced. Analysis of the g-ratios by three-way ANOVA followed with Holm-Sidak post hoc analysis for pair-wise comparisons indicated a significant difference in g-ratio of small and large motor axons between wild type and NF-M\textsuperscript{BovineTail} mice at 2 and 6 months.
Figure 4.7. Motor nerve conduction velocity and myelin structure was unaltered in NF-M\textsuperscript{BovineTail} mice. Motor nerve conduction velocity (MNCV) was measured from axons of the sciatic nerve in 6-month-old wild type and NF-M\textsuperscript{BovineTail} mice (A). Despite increased axonal diameters, MNCV in wild type and NF-M\textsuperscript{BovineTail} mice were indistinguishable. Average conduction velocities were analyzed by Student’s t-test. There was not a statistically significant difference in conduction velocity between wild type and NF-M\textsuperscript{BovineTail} mice. Error bars = SEM. N= 7. Schematic of measurements (B) utilized to calculate g-ratios (axon diameter/fiber diameter) of 10% of all myelinated axons from L5 motor roots in five mice per genotype and age group reported in Table 1. Bottom left, representative EM images of myelin from L5 small and large motor axons of 6-month wild type and NF-M\textsuperscript{BovineTail} mice (C); Scale bar = 1 µm. Bottom right, images of myelin lamellae from small and large motor axons; Scale bar = 50 nm. Myelin structure was unaltered NF-M\textsuperscript{BovineTail} mice relative to wild type.
Table 4.1. Myelin thickness and g-ratios were altered in motor axons of NF-M\textsuperscript{BovineTail} mice.

<table>
<thead>
<tr>
<th>Axon</th>
<th>Genotype</th>
<th>Age (months)</th>
<th>Peak axonal diameter (µm)</th>
<th>Average Myelin thickness (µm)</th>
<th>Average g-ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small Motor (&lt; 4 µm)</td>
<td>wild type</td>
<td>2</td>
<td>1.5</td>
<td>0.730 ± 0.013</td>
<td>0.586 ± 0.004</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>1.5</td>
<td>0.826 ± 0.016</td>
<td>0.576 ± 0.004</td>
</tr>
<tr>
<td></td>
<td>NF-M\textsuperscript{BovineTail}</td>
<td>2</td>
<td>1</td>
<td>0.690 ± 0.012*</td>
<td>0.604 ± 0.004*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>1.5</td>
<td>0.768 ± 0.015*</td>
<td>0.604 ± 0.004**</td>
</tr>
<tr>
<td>Large Motor (&gt; 4 µm)</td>
<td>wild type</td>
<td>2</td>
<td>5</td>
<td>1.442 ± 0.015</td>
<td>0.707 ± 0.003</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>7.5</td>
<td>1.754 ± 0.014</td>
<td>0.724 ± 0.003</td>
</tr>
<tr>
<td></td>
<td>NF-M\textsuperscript{BovineTail}</td>
<td>2</td>
<td>6</td>
<td>1.423 ± 0.015</td>
<td>0.719 ± 0.003**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>8</td>
<td>1.690 ± 0.013**</td>
<td>0.741 ± 0.003**</td>
</tr>
</tbody>
</table>

\(g\)-ratio = axon diameter/ fiber diameter (myelin and axon diameter); ± = SEM; * \(p< 0.05\); ** \(p< 0.001\)
Inspection of compact myelin of small and large motor axons indicated no observable differences in myelin structure (Fig 4.7C).

Mathematical modeling was performed to predict the effect of altering axonal diameter and g-ratios on conduction velocity in NF-M\textsuperscript{BovineTail} mice (Table 2), bearing in mind that our experimental MNCV measurements record the velocity of large myelinated fibers. Consistent with experimental MNCV results, the modeling predicted a MNCV of 46.8 m/s, which was similar to the MNCV measured in NF-M\textsuperscript{BovineTail} mice. Interestingly, if g-ratios were the same in NF-M\textsuperscript{BovineTail} mice as wild type, the model predicted an increase in MNCV to 50.5 m/s in NF-M\textsuperscript{BovineTail} mice (Table 2).

Discussion:

NF-M C-terminus mediated radial growth by a mechanism that was independent of KSP phosphorylation. Using a combination of gene targeting and computational modeling, we demonstrated that increasing the overall length of NF-M C-terminus resulted in larger axonal diameters in motor neurons. Increasing the length of NF-M C-terminus resulted in an increase in peak axonal volume of ~44% percent at 2 months and ~22% at 6 months relative to wild type. The enhancement at 2 months was exactly the same magnitude observed in mice overexpressing NF-L and NF-M subunits by 300 and 360 percent, respectively, relative to wild type levels (Xu et al., 1996). However, enhanced radial growth in NF-M\textsuperscript{BovineTail} mice was accomplished by expanding the NF-M C-terminus by the addition of 15 KSP repeats and 30 non-KSP repeat amino acids.
Table 4.2. Reductions in myelin thickness were predicted to be sufficient to prevent increased motor nerve conduction velocity in NF-M<sup>BovineTail</sup> mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>Diameter (µm)</th>
<th>G-ratio</th>
<th>R&lt;sub&gt;m&lt;/sub&gt; (MΩ cm&lt;sup&gt;2&lt;/sup&gt;)</th>
<th>R&lt;sub&gt;i&lt;/sub&gt; (Ω cm)</th>
<th>C&lt;sub&gt;m&lt;/sub&gt; (µF cm&lt;sup&gt;-2&lt;/sup&gt;)</th>
<th>τ&lt;sub&gt;m&lt;/sub&gt; (ms)</th>
<th>MNCV (m/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type mouse</td>
<td>7.5</td>
<td>0.72</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>47.3 ± 2.6</td>
</tr>
<tr>
<td>NF-M&lt;sup&gt;BovineTail&lt;/sup&gt; mouse</td>
<td>8</td>
<td>0.74</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>46.2 ± 1.7</td>
</tr>
<tr>
<td>wild type model</td>
<td>7.5</td>
<td>0.72</td>
<td>25.8</td>
<td>200</td>
<td>0.6</td>
<td>1.55</td>
<td>47.2</td>
</tr>
<tr>
<td>NF-M&lt;sup&gt;BovineTail&lt;/sup&gt; model (Δ diameter + Δ g-ratio)</td>
<td>8</td>
<td>0.74</td>
<td>25.8</td>
<td>200</td>
<td>0.646</td>
<td>1.67</td>
<td>46.8</td>
</tr>
<tr>
<td>NF-M&lt;sup&gt;BovineTail&lt;/sup&gt; model (Δ diameter)</td>
<td>8</td>
<td>0.72</td>
<td>25.8</td>
<td>200</td>
<td>0.6</td>
<td>1.55</td>
<td>50.5</td>
</tr>
</tbody>
</table>

R<sub>m</sub> = membrane resistance, R<sub>i</sub> = axonal resistance, C<sub>m</sub> = membrane capacitance, τ<sub>m</sub> = membrane time constant, MNCV = motor nerve conduction velocity, Δ diameter = change in diameter observed experimentally, Δ g-ratio = change in g-ratio observed experimentally, ± = SEM.
This alteration did not affect subunit stoichiometry, nor did it affect NF-M interactions with other NF subunits. The diameter of small motor axons was initially decreased at 2 months. However, by 6 months, small motor axons were indistinguishable from wild type littermates.

Increasing the length of NF-M C-terminus may have enhanced radial growth by increasing the distance the C-terminus extended from the filament core. Utilizing parameters originally developed for wild type mouse and human NF-M (Chang et al., 2009; Stevenson et al., 2011), computational analysis of NF-M\textsuperscript{BovineTail} suggested that the longer C-terminus projected farther from the filament core than wild type mouse NF-M in conditions that simulate the ionic strength of axoplasm. Moreover, increased extension was not dependent upon the phosphorylation status of NF-M C-terminal KSP repeats supporting the observation that expressing KSP phospho-incompetent NF-M did not prevent radial growth in vivo (Garcia et al., 2009). Despite potentially extending farther from the filament core, axoplasmic organization and NF-NF spacing were unaltered in NF-M\textsuperscript{BovineTail} motor or sensory axons. These results supported earlier observation that NF-NF spacing did not directly correlate with larger axonal diameters (Garcia et al., 2003; Garcia et al., 2009).

Despite increased axonal diameter, motor nerve conduction velocity (MNCV) of the largest fibers in the sciatic nerve was unaffected in NF-M\textsuperscript{BovineTail} mice (Fig. 4.7A). Failure to increase conduction velocity in motor axons may have resulted from relatively thinner myelin in NF-M\textsuperscript{BovineTail} mice, without observable differences in the structure of myelin lamellae. Comparing myelin
thickness at 2 and 6 months indicated that NF-M\textsuperscript{BovineTail} mice did not increase their myelin thickness in response to larger axonal diameters. Mathematical modeling of conduction velocity suggested that the observed failure to adequately increase myelin thickness was sufficient to account for slower than expected conduction velocities in NF-M\textsuperscript{BovineTail} mice. Moreover, mathematical modeling indicated that, had myelin thickness increased in proportion with increasing axonal diameter in NF-M\textsuperscript{BovineTail} mice, the observed increase in fiber diameter would have resulted in faster MNCV. Despite not increasing small motor axon diameter, expansion of NF-M C-terminus resulted in a significant reduction in relative myelin thickness and a significant increase in g-ratio of small motor axons. In fact, the increased g-ratio of small motor axons was greater than the observed increase in large motor axons. A more pronounced effect on myelin thickness was also observed in small axons of mice with altered neuregulin (NRG) expression (Michailov et al., 2004). It is unclear why small axons were more susceptible to genetic manipulations that altered myelin thickness.

Increased expression of NRG1 type III resulted in significantly thicker myelin sheaths (Michailov et al., 2004), but axonal diameter appeared largely unaffected. Moreover, increasing radial growth, by expanding the length of NF-M C-terminus, resulted in significantly larger motor axons with myelin sheaths that failed to expand proportionally. Active signaling has been observed between Schwann cells and axons. NRG1 type III was an axonal ligand for the Schwann cell receptor, ErbB2, (Nave and Salzer, 2006) that regulated myelin thickness (Michailov et al., 2004). Formation of compact myelin increased NF
phosphorylation (de Waegh et al., 1992) possibly through the interaction of myelin associated glycoprotein (Yin et al., 1998) with an as yet to be identified axonal receptor. However, when taken together with the NRG1 type III results, our data suggested that once the processes of myelination and radial growth initiated there was no active feedback between myelinating Schwann cell and axons. Therefore, during myelination and radial growth, there was no plasticity allowing for compensatory alterations to myelin thickness or axonal diameter due to enhanced radial growth or myelination, respectively.
Appendix to Chapter 4: Analysis of Sensory Axons in NF-M^{BovineTail} mice

NF-M^{BovineTail} expression did not affect radial growth of sensory axons.

Previous analyses of NF gene deleted mice suggested that NF-M and NF-H subunit expression influences radial growth of peripheral sensory axons (Elder et al., 1999; Rao et al., 1998). Therefore, axonal diameters were measured from cross sections of the 5th lumbar sensory root (Fig. 4.8A). Survival of sensory axons was unaffected at both 2 and 6 months (Fig. 4.8B). Radial growth of sensory axons in NF-M^{BovineTail} mice was largely unaffected at both 2 (Fig. 4.8C) and 6 (Fig. 4.8D) months as peak diameters and overall distributions were indistinguishable when compared to wild type mice. The number of NF-M^{BovineTail} sensory axons was unaffected relative to wild type littermates at 2 and 6 months (Fig. 4.8B).

Expression of NF-M^{BovineTail} did not affect neurofilament spacing or clustering in sensory axons.

NF spacing and number were quantified by calculating nearest neighbor distances (NND) of adjacent NFs within axoplasm of sensory axons at 2 (Fig. 4.9A) and 6 (Fig. 4.9C) months. The peak in NF spacing was unaffected in NF-M^{BovineTail} sensory axons when compared to wild type. Peak distances of NFs in wild type and NF-M^{BovineTail} sensory axoplasm were identical at 2 and 6 months. NF number was reduced in sensory axons at 2 months but the decrease was not
Figure 4.8. Sensory axon radial growth was unaffected in NF-M\textsuperscript{BovineTail} mice. Transverse sections of L5 sensory (dorsal) root axons from wild type and NF-M\textsuperscript{BovineTail} homozygous mice (A). Scale bar = 10µm. Number of axons in L5 sensory roots from 2 and 6-month-old wild type and NF-M\textsuperscript{BovineTail} mice (B). Counts are average from five animals for each genotype and age group. Distribution of axonal diameters in sensory axons of 2 (C) or 6 (D) month-old wild type and NF-M\textsuperscript{BovineTail} mice. Diameter distributions are unaltered in sensory axons of NF-M\textsuperscript{BovineTail} mice. Points represent the averaged distribution of axon diameters from the entire roots of five mice for each genotype and age group. N=5.
Figure 4.9. Neurofilament spacing was unaffected in sensory axons of NF-M<sup>BovineTail</sup> mice. Distribution of nearest-neighbor distances (NND) was determined for sensory axons from wild type and NF-M<sup>BovineTail</sup> mice at 2 (A) and 6 (C) months. Additionally, NF numbers were determined for sensory axons from wild type and NF-M<sup>BovineTail</sup> mice at 2 (B) and 6 (D) months. Peak NND was unaffected for sensory axons of NF-M<sup>BovineTail</sup> mice at either time point. Moreover, NF number in small and large motor axons was not significantly affected in NF-M<sup>BovineTail</sup> mice at either time point. Points represent averaged frequencies of NF nearest neighbor distances in axons from five mice per genotype and age group. Average number of neurofilaments was analyzed for statistical significance by Student’s t-test. NF clustering was unaffected in sensory axons in NF-M<sup>BovineTail</sup> mice at 2 and 6 months (E). NF densities (F) tended to be reduced in sensory axons at 6 months in NF-M<sup>BovineTail</sup> mice. However, the differences were not statistically significant. Averages were analyzed for statistical significance by two-way ANOVA. Error bars = SEM. N= 5.
statistically significant (Fig. 4.9B). NF number was unaltered in sensory axons at 6 months (Fig. 4.9D).

Neurofilament clustering was measured by identifying the positions of all NFs in sensory axons and calculating the ratio of average filament spacing to nearest neighbor distance. In sensory axons, NF clustering indexes were slightly less in NF-M\textsuperscript{BovineTail} at 2 and 6 months (Fig. 4.9E) relative to wild type suggesting a more ordered array of NFs. However, statistical analysis by two-way ANOVA indicated the difference in NF clustering between wild type and NF-M\textsuperscript{BovineTail} was not significant. NF densities (number of NFs/ µm\textsuperscript{2} of axoplasm) were unaffected at 2 months in NF-M\textsuperscript{BovineTail} sensory axons (Fig. 4.9F). NF densities in NF-M\textsuperscript{BovineTail} sensory axons tended to be reduced at 6 months but the differences were not significantly different between wild type and NF-M\textsuperscript{BovineTail} mice (Fig. 4.9F).

**Appendix to Chapter 4 Conclusions:**

Analysis of sensory axonal diameters suggested that expansion of the NF-M C-terminus did not affect radial growth of sensory axons. Previous evidence suggested that truncation of the NF-M C-terminus in mice (NF-M\textsuperscript{TailΔ}) reduced radial growth of large sensory axons (Rao et al., 2003), yet our analysis in NF-M\textsuperscript{BovineTail} mice did not detect any observable differences in radial growth of small or large sensory axons. Moreover, a recent analysis of sensory axons in NF-M\textsuperscript{TailΔ} mice indicated that the NF-M C-terminus did not affect radial growth of sensory axons but affected survival of sensory axons (Downer et al., in
preparation). In the absence of the NF-M C-terminus, survival was significantly reduced (Downer et al., in preparation). In NF-M$^{\text{BovineTail}}$ mice the NF-M C-terminus was expanded, not absent, and survival was unaffected. Taken together, the NF-M C-terminus, independent of its length, allowed for normal survival of sensory axons. Analysis of radial growth in motor and sensory axons in NF-M$^{\text{TailA}}$ and NF-M$^{\text{BovineTail}}$ mice suggested that the NF-M C-terminus might have different effects on neurons derived from different tissues. In motor axons, the NF-M C-terminus determined radial growth but did not affect survival. Conversely, the NF-M C-terminus did not affect radial growth but was important for survival in sensory axons.
CHAPTER 5
CONCLUSIONS

Signaling within the nervous system occurs from and to the periphery of organisms over long distances. Due to the distance that the signals must travel, rapid signaling is necessary for organisms to adapt quickly. Vertebrate neurons conduct signals rapidly due to axonal myelination (Huxley and Stampfli, 1949). Myelination is a process by which glial cells of the nervous system ensheath the axon with concentric layers of membrane known as compact myelin. The myelin insulates the axon to prevent charge loss and reduces capacitance along the axon contributing to increased rate of signal conduction (Koles and Rasminsky, 1972; Rasminsky and Sears, 1972). Myelination also induces the axon to undergo radial growth only within myelinated regions, which increases the efficiency by which charge spreads along the axon during signal conduction (Hursh, 1939; Rushton, 1951). Radial growth of myelinated axons is, therefore, an important developmental process that allows for fast signaling in the mature nervous system.

Neurofilaments and the formation of compact myelin were both required for radial axonal growth (de Waegh et al., 1992; Ohara et al., 1993; Sakaguchi et al., 1993; Zhu et al., 1997). NF-M and NF-H subunits were more heavily phosphorylated in myelinated regions of axons relative to unmyelinated regions of the same axon (de Waegh et al., 1992; Hsieh et al., 1994; Starr et al., 1996; Yin et al., 1998). It was, therefore, hypothesized that myelination regulated NF
phosphorylation which in turn influenced radial growth of axons (Cleveland, 1996). According to the hypothesis, the more heavily phosphorylated NF-H was predicted to be the critical subunit for radial growth. Deletion of NF-H resulted in various magnitudes of reductions in radial growth resulting in axonal diameters that were smaller than wild type mice (Elder et al., 1998b; Rao et al., 1998; Zhu et al., 1998). However, all observed reductions in radial growth were less than predicted. NF-M deletion resulted in reductions in radial growth resulting in axonal diameters that were similar in size to axons completely devoid of all neurofilaments (Elder et al., 1998a; Zhu et al., 1997). Taken together, these data suggested that NF-M was the critical subunit for radial axonal growth.

Radial growth was analyzed in mice expressing C-terminally truncated NF-H and NF-M, which had the advantage of not altering subunit stoichiometry inherent to classic gene deletion studies. Analysis of radial growth in mice expressing C-terminally truncated NF-H suggested that the longer and more heavily phosphorylated NF-H C-terminus was not important for overall radial growth (Garcia et al., 2003; Rao et al., 2002). Moreover, analysis of radial growth in mice expressing C-terminally truncated NF-M suggested that NF-M C-terminus was essential for radial growth (Garcia et al., 2003; Rao et al., 2003). Mechanistically, it was hypothesized that truncation of the entire NF-M C-terminus deleted essential phosphorylation sites that occurred on highly conserved lysine-serine-proline (KSP) repeats (Garcia et al., 2003; Rao et al., 2003). However, preventing NF-M KSP phosphorylation in mice did not prevent radial growth (Garcia et al., 2009). Therefore, the role of NF-H and the
mechanism by which NF-M C-terminus regulated radial growth remained unclear. In my dissertation, I have contributed new insights that put developmental delay of NF-H expression into context with radial growth along the length of peripheral nervous system axons. Moreover, my work presented a novel mechanism by which the NF-M C-terminus regulated radial growth in large motor axons.

5.1 Distal to proximal development of axons requires NF-H expression

Neurofilament phosphorylation was hypothesized to regulate radial growth in myelinated regions of axons. Based on this hypothesis, the more heavily phosphorylated NF-H subunit was predicted to be critical for radial growth. However, deletion of NF-H resulted in reductions in radial growth that were less than expected (Elder et al., 1998b; Rao et al., 1998; Zhu et al., 1998). For 13 years, no further analysis of NF-H was performed to better understand its role in radial growth. Moreover, previous studies were limited to analysis of radial growth in only proximal regions of axons (fifth lumbar nerve roots). To gain further insight into the role of NF-H, I analyzed radial growth at distal and proximal segments of the sciatic nerve and along the entire length of the phrenic nerve. My analysis of radial growth in these two nerves suggested that radial growth of axons and NF number were reduced in proximal segments of peripheral nerves in NF-H⁺/− mice. Yet, radial growth and NF number were unaffected in the most distal segments of peripheral nerves in NF-H⁺/− mice. Taken together, these observations suggested that NF-H expression was required for proximal postnatal development of peripheral nervous system axons.
To provide new insights into the potential role of NF-H, my interpretation of these new NF-H observations was made with a different perspective versus the original reports (Elder et al., 1998b; Rao et al., 1998; Zhu et al., 1998), which allowed me to tie together the following observations made about NF-H:


2. NF transport slowed during postnatal development (Hoffman et al., 1983) and coincided with increased expression of NF-H (Pachter and Liem, 1984; Shaw and Weber, 1982).

3. Radial growth also correlated temporally with a slowing of NF transport (Hoffman et al., 1985a; Hoffman et al., 1984; Hoffman et al., 1985b).

Due to the developmental delay in NF-H expression, I concluded that NF-H

mice contained NFs that were similar in composition to early developmental stages. Collectively, these data suggested that prior to increased NF-H expression, NFs were primarily composed of NF-L and NF-M, were transported at a faster rate and accumulated in distal axonal segments. As NF-H expression increased, the rate of NF transport slowed allowing NFs to accumulate and radial growth to increase in proximal axonal segments. Taken together with my observations, the data suggested a new model for radial growth. NF-H expression remained low during embryonic and early postnatal development to
allow distal axonal segments to develop prior to proximal segments (Fig. 5.1). The role of NF-H was, therefore, to allow proximal accumulation of NFs and subsequent development of proximal axonal segments. NF-H<sup>−/−</sup> mice might serve as a model for early postnatal development of peripheral axons.

Mechanistically, development of distal axonal segments prior to proximal axonal segments might have been important for the long-term maintenance of radial growth along the axon. Previous evidence indicated that NFs were long lived proteins in regions of axons with established NF networks such as in regions that have undergone radial growth (Millecamps et al., 2007; Yuan et al., 2009). However, in axonal regions in which NF networks were less established and had not yet undergone radial growth, NF turnover was increased (Millecamps et al., 2007; Yuan et al., 2009). It would, therefore, be advantageous to allow NFs to accumulate and radial growth to occur in distal axonal segments early in development when distal segments of axons were still relatively close to the cell body. This would be especially important for larger mammals in which distal segments of peripheral nerves become further removed from the cell body as development progresses. If proximal axonal segments developed first, subsequent development of distal axonal segments might be problematic due to slowing of NF transport through proximal segments, increased NF turnover rate in distal axonal segments, and increased peripheral axon length as development progresses. Moreover, if distal axonal segments failed to undergo radial growth, conduction rates would likely decrease which may be detrimental for the survival of the organism. With distal to proximal
Figure 5.1. Distal segments of axons developed prior to proximal segments due to delayed NF-H expression. Early in postnatal development (top neuron), NF-H expression remained low relative to NF-L and NF-M (indicated by arrows). With NF-H expression low, NF transport rate was increased (indicated by arrow) resulting in NF accumulation and initiation of radial growth in distal segments. As postnatal development progressed (middle neuron), NF-H expression increased resulting in decreased NF transport rate allowing for NF accumulation and radial growth to occur in more proximal axonal segments. In the later stage of postnatal development (bottom neuron) NF-H expression increased to adult levels resulting in further decreases in NF transport rate and radial growth to occur in the most proximal segments.
development, distal axonal diameter would be established early in development and would be maintained due to reduced NF turnover rates allowing NFs to be maintained as long lived proteins. Subsequent development of proximal axonal segments would allow for the establishment of a uniform axonal morphology and neuronal conduction velocity, which would likely benefit the organism’s chances of survival.

5.2 Variation of the NF-M KSP repeat sub-domain may have been a molecular mechanism to increase radial growth during mammalian evolution

The evolution of larger mammals resulted in increased axonal length. I hypothesized that selective pressures resulted in increased conduction velocities in larger mammals with longer axons. Increased conduction velocity in longer axons would maintain the latency at which axons signal to their prospective targets. If indeed conduction velocities were increased as larger mammals evolved, it is probable that the primary cell biological properties that influence conduction velocity, radial axonal growth and myelination, were likely the targets of selective pressure.

Deletion of the NF-M C-terminus in mice suggested that the NF-M C-terminus was essential for radial growth of large motor axons (Garcia et al., 2003; Rao et al., 2003). Loss of the NF-M C-terminus in mice resulted in a 40% reduction in radial growth of large motor axons and a 30% reduction in motor nerve conduction velocity relative to wild type mice (Garcia et al., 2003). These
findings suggested that the NF-M C-terminus might have been a target of selective pressure as it was a determinant of radial axonal growth and, consequently, conduction velocity. However, the mechanism by which the NF-M C-terminus would have responded to selective pressure to regulate radial growth in evolving mammals remained unclear. To gain insight into the function of the NF-M C-terminus in mediating radial growth, I characterized the NF-M C-terminus through phylogenetic sequencing and morphological analyses of axons across mammals.

Sequencing analysis of 26 mammalian species revealed that the C-terminus of NF-M was actually composed of three sub-domains, two conserved domains flanking a variable region. Within the variable region of NF-M C-terminus, KSP repeat number was highly variable across species yet did not vary within species. Linear regression analyses indicated that NF-M KSP repeat number positively correlated with head-body length, a measurement that was an indicator of axonal length. For a subset of mammals, KSP repeat number positively correlated with axonal diameter in large motor axons. These data suggested the variations in KSP repeat number were not random but varied due to selective pressures, possibly in order to regulate radial growth and conduction velocity. Variation of a repetitive region such as the KSP repeat region, possibly through DNA polymerase slippage events, would have been a relatively straightforward mechanism for altering NF-M C-terminal function as mammals evolved. Previous evidence indicated that variation in length of tandem repeats within coding regions of several genes correlated with robust changes in limb and
skull morphologies in dogs (Fondon and Garner, 2004). Variation of NF-M KSP repeats might have, therefore, been a mechanism to adjust axonal morphology as axonal length varied during mammalian evolution.

NF-M KSP repeat number varied considerably across mammals yet sequencing within species revealed no detectable variation of KSP repeats or any other amino acids of the NF-M C-terminus within species. This suggested that NF-M C-terminus and KSP repeat number was stabilized for each species during evolution. Mammals maintained a level of radial growth optimal for their respective axonal length by stabilizing the number of KSP repeats. For example, mice that have much shorter axons would not have required large increases in radial growth and so KSP repeat number was stabilized to a relatively low number. Conversely, chimps have longer axons and would have required increased radial growth, but not to the level of extremely long axons, such as in elephant. KSP repeat number in chimps was, therefore, maintained at a number sufficient, but not excessive, to increase radial growth to the level required to increase conduction velocity proportionally with axonal length.

It was not feasible to obtain nerve conduction velocity measurements from the mammals of the sequencing analysis. However, direct measurements of nerve conduction velocities in several species including elephant (More et al., 2010), sheep (Loke et al., 1986), pig (Szentkuti et al., 1990), human (Chang et al., 2006) and mouse (Garcia et al., 2003; Garcia et al., 2009) suggested that larger mammals have faster rates of conduction. Moreover, conduction velocity recordings from cat hind limb fibers demonstrated that larger diameter fibers
have faster conduction velocities (Hursh, 1939) and was supported in subsequent studies (Arbuthnott et al., 1980b; Boyd and Kalu, 1979; Westbury, 1982), as well as theoretical results (Rushton, 1951; Smith and Koles, 1970; Waxman, 1980). The results from my analysis suggested that increased length of NF-M, through expanded number of KSP repeats, was associated with larger axonal diameters. Taken together, expanding the NF-M C-terminus length may have been a mechanism to increase axonal diameter and conduction velocity in larger mammals. However, in a second subset of larger mammals the number of KSP repeats remained low and did not correlate with head-body length. Horse and dog NF-M KSP repeats were low yet MNCV recordings indicated faster rates of conduction in horse (Henry et al., 1979) and dog (Walker et al., 1979) motor axons relative to MNCV recording in mouse (Garcia et al., 2003) motor axons. It is probable that NF-M KSP repeat expansion might have not been the only evolutionary mechanism to increase radial growth and conduction velocity as larger mammals evolved.

The data also suggested a new hypothesis for NF-M C-terminus in determining radial growth of motor axons. The previous hypothesis that NF C-terminal phosphorylation regulated radial growth in myelinated regions of axon was systematically disproven through several lines of NF gene-replacement mice (Garcia et al., 2003; Garcia et al., 2009; Rao et al., 2003; Rao et al., 2002). My work supported a new hypothesis that suggested the length of the NF-M C-terminus, through KSP repeat variation, regulated the magnitude of radial growth in motor axons as subsets of mammalian species evolved.
5.3 Expansion of the NF-M C-terminus in mice increases radial growth independent of increases in conduction velocity or myelin thickness.

Myelination was hypothesized to regulate radial growth through an “outside-in” signal that initiated with formation of compact myelin and that resulted in increased NF-M KSP phosphorylation (Garcia et al., 2003). Increased NF-M KSP phosphorylation was thought to determine axonal diameter through increased NF-NF spacing due to increased negative charge density. However, this hypothesis was disproven as prevention of NF-M KSP phosphorylation did not result in reduction in radial axonal growth (Garcia et al., 2009). Therefore, the mechanism by which NF-M determined radial axonal growth remained unclear. Based upon the phylogenetic sequencing analyses that I performed, I proposed a new hypothesis that the length of the NF-M C-terminus, through KSP repeat variation, was the mechanism by which NF-M regulated radial growth of motor axons. However, the analyses I performed only had the power to correlate the number of NF-M KSP repeats, and therefore C-terminal length, with axonal diameter. Moreover, the sample size was limited due to difficulties obtaining DNA and nervous tissue.

To provide a direct test of the new hypothesis, I generated a novel line of gene replacement mice that expressed NF-M in which the length of the C-terminus was increased. Replacing the endogenous NF-M C-terminus with the longer bovine C-terminus (NF-M\textsuperscript{BovineTail} mice) increased the overall length of NF-M C-terminus by 76 amino acids as well as 15 additional KSP repeats. The expanded NF-M C-terminus increased radial growth of large motor axons at both
2 and 6 months as peak diameter was increased relative to wild type mice. However, radial growth in the NF-M$^{\text{BovineTail}}$ mice did not increase axonal diameter to the level that was observed in bovine motor axons. This suggested that other factors, such as body size constraints, might have limited the increase in radial growth in mice with expanded NF-M C-terminus. Despite these limiting factors, expanding the NF-M C-terminus, through additional KSP repeats, increased radial growth of motor axons in mice.

My analysis suggested that expansion of the NF-M C-terminus increased radial growth. Taken together with previous evidence, the data supported a new model for NF-M C-terminus dependent radial growth. The length of the NF-M C-terminus determined the magnitude of radial growth of large motor axons (Fig. 5.2). In the absence of the NF-M C-terminus, minimal radial growth occurred in large motor axons, and axonal diameter was reduced relative to wild type mice. Expanding the length of the NF-M C-terminus (NF-M$^{\text{BovineTail}}$) resulted increased radial growth in large motor axons relative to wild type axons. The generation and analysis of NF-M$^{\text{BovineTail}}$ mice supported the hypothesis that increasing the length of the NF-M C-terminus through variation of the KSP repeat sub-domain may have been a mechanism to increase axonal diameter as larger mammals evolved.

Despite the increase in diameter of large motor axons, motor nerve conduction velocity (MNCV) in NF-M$^{\text{BovineTail}}$ mice did not increase relative to wild type. Analysis of myelin sheaths indicated that myelin thickness did not increase proportionally with the increase in motor axon diameter as g-ratios of large motor
Figure 5.2. The length of the NF-M C-terminus determined the magnitude of radial growth of large motor axons. Removal of the NF-M C-terminus (top, motor neuron from NF-M^tail mice) resulted in minimal radial growth in myelinated regions of axon (Garcia et al., 2003). Radial growth of large motor axons was reduced in the absence of the NF-M C-terminus relative to wild type (middle, wild type motor neuron). Increasing the length of the NF-M C-terminus in NF-M^{BovineTail} mice (bottom) increased radial growth of large motor axons relative to wild type. Taken together, the NF-M C-terminal length determined the magnitude of radial axonal growth in large motor axons.
axons were increased in NF-M\textsuperscript{BovineTail} mice. Failure of myelin thickness to increase proportionally with increased axonal diameter was predicted to be sufficient to prevent an increase in MNCV in NF-M\textsuperscript{BovineTail} mice. Taken together, the data suggested that increasing conduction velocity in the peripheral nervous system cannot be accomplished simply through affecting one component, such as axonal diameter. The peripheral nervous system is complex and requires concerted increases in both myelin thickness and radial axonal growth in order to increase the rate of conduction along myelinated axons. It was probable that during mammalian evolution, myelin thickness was increased concomitantly with increases in axonal diameter in order maintain optimal g-ratios and increase conduction velocity as larger mammals evolved. Myelin thickness could have increased through increasing expression levels of Neuregulin 1 (Nrg1) type III. Overexpression of Nrg1 type III in mice resulted in increased myelin thickness without increased axonal diameter (Michailov et al., 2004). Increasing nerve conduction velocity as larger mammals evolved might have, therefore, been accomplished through 1) increasing axonal diameter through NF-M C-terminus expansion via KSP repeat variation and 2) increasing myelin thickness through increasing Nrg1 type III expression levels. This would have resulted in increasing the diameter of myelinated fibers while maintaining the normal proportion of axonal diameter to fiber diameter (g-ratio) in large motor axons.

Analysis of large motor axons suggested a lack of feedback in the processes of myelination and radial axonal growth. However, analysis of small motor axons indicated a decrease in myelin thickness despite no significant
difference in small motor axonal diameter distribution in NF-M\textsuperscript{BovineTail} mice. This result suggested that myelination and radial growth in small motor axons might contain a feedback mechanism from axon to Schwann cell that negatively regulates the process of myelination. It remained unclear the mechanism by which expansion of the NF-M C-terminus within the axon could result in changes in myelination. As a component of the axonal cytoskeleton, the NF-M C-terminus might play a role to integrate a putative feedback signaling complex that regulates Schwann cell myelination in small motor axons. Synapse-associated protein (SAP) 90/Postsynaptic density (PSD)-95-associated protein (SAPAP) interacts with NF-M and other cytoskeletal proteins to provide scaffolding for components important for synaptic function (Hirao et al., 2000). NF-M and its C-terminus could potentially play a similar scaffolding role along the axon to allow for regulation of myelination through a putative signaling complex.

Innervation of target muscle fiber types and muscle fiber target size could also potentially play a role in the differences in myelination and radial growth of small and large motor axons. Small motor axons innervate muscle spindles whereas large motor axons innervate extrafuscle muscle fibers. Increasing postsynaptic target size resulted in increased fiber diameter (increased both myelin thickness and axonal diameter) (Elashry et al., 2011; Voyvodic, 1989) suggesting that a putative signal from the muscle target could regulate the processes of myelination and radial axonal growth. Differences in gene expression might also contribute to the differences in myelination and radial growth between small and large motor axons. Small and large motor neurons
express transcriptional regulatory proteins that are distinct from one another (Friese et al., 2009). It will be important to further characterize the relationship between myelinated fiber and postsynaptic target in mediating the processes of myelination and radial growth of axons.

Mechanistically, it remained unclear how the expansion NF-M C-terminus mediated an increase in radial growth. Previous evidence suggested that NF C-terminal projections formed cross-bridge structures with adjacent NFs (Hirokawa et al., 1984; Hisanaga and Hirokawa, 1988). Mathematical modeling of mouse and bovine NF-M C-termini indicated that the expansion of the C-terminus increased lateral extension from the filament core. Increasing the lateral extension of NF-M C-termini through expansion of the NF-M C-terminus might have extended the cross-bridge length, allowing for longer range interactions between NFs that provide greater structural stability for larger diameter axons.

Expansion of the NF-M C-terminus might have enhanced interactions with putative cytolinker proteins that synergistically mediated an increase in axonal diameter of large motor axons. Cytolinker proteins such as plakins are capable of binding multiple cytoskeletal elements including intermediate filaments (Jefferson et al., 2004). Plectin, a plakin family member protein, mediated interactions of intermediate filaments with microtubules and actin in fibroblasts (Svitkina et al., 1996). The neuronal isoform of plectin, plectin 1c, is expressed in motor neurons (Errante et al., 1994). Targeted deletion of plectin 1c in mice resulted in reduced diameters of large axons and reduced nerve conduction velocity (Fuchs et al., 2009) suggesting a potential interaction with
neurofilaments in mediating radial axonal growth. However, there have been no confirmed interactions between plectin and motor neuron derived neurofilaments. Identification of NF interacting proteins will be important in further elucidating the mechanism by which the axonal cytoskeleton mediates radial growth of large motor axons.

In summary, my dissertation has provided new understanding into the role of both NF-H and NF-M subunits in the process of radial axonal growth. NF-H expression was developmentally delayed to allow distal regions of axons to develop prior to proximal axonal regions, which may have been important for long-term maintenance of axonal diameter in the adult organism. The variation in NF-M KSP repeats and length of the NF-M C-terminus may have been a molecular mechanism to increase radial growth of motor axons as larger mammals evolved. Expansion of the NF-M C-terminus in mice increased radial growth of large motor axons independent of increases in conduction velocity or myelin thickness. Taken together with previous data, my work suggested that the length of the NF-M C-terminus determined the magnitude of radial growth in large motor axons.
References:


design and implementation of the UniProt website. *BMC Bioinformatics.* 10:136.


VITA

I was born in Kansas City, Missouri January 19, 1984 but grew up in Lathrop, Missouri. Lathrop is a rural town outside of Kansas City and my father was the dentist in town. My mother worked with my father as a hygienist. I have two younger brothers with whom I am very close. I gravitated toward science and mathematics at an early age and participated in many academic activities including Math Contest and Science Olympiad in elementary and junior high school. In high school, I had a great biology and chemistry teacher who gave me a base of scientific knowledge and gave me the opportunity to take college courses in high school.

My initial interests were primarily in chemistry and mathematics and I enrolled in college with chemistry as my major. However, in my sophomore year I realized my true interest was in biology. I pursued biology as my major and graduated with a Bachelor of Sciences at the University of Missouri in 2006. After one year as a research technician, I entered the graduate program at the University of Missouri. During graduate school I married my wife, Rachel, and we had a son, Gavin. I earned my Doctor of Philosophy in Biological Sciences at the University of Missouri in 2012.