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Research paper

# Internode length is reduced during myelination and remyelination by neurofilament medium phosphorylation in motor axons

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ABSTRACT

The distance between nodes of Ranvier, referred to as internode length, positively correlates with axon diameter, and is optimized during development to ensure maximal neuronal conduction velocity. Following myelin loss, internode length is reestablished through remyelination. However, remyelination results in short internode lengths and reduced conduction rates. We analyzed the potential role of neurofilament phosphorylation in regulating internode length during remyelination and myelination. Following ethidium bromide induced demyelination, levels of neurofilament medium (NF-M) and heavy (NF-H) phosphorylation were unaffected. Preventing NF-M lysine-serine-proline (KSP) repeat phosphorylation increased internode length by 30% after remyelination. To further analyze the role of NF-M phosphorylation in regulating internode length, gene replacement was used to produce mice in which all KSP serine residues were replaced with glutamate to mimic constitutive phosphorylation. Mimicking constitutive KSP phosphorylation reduced internode length by 16% during myelination and motor nerve conduction velocity by ~27% without altering sensory nerve structure or function. Our results suggest that NF-M KSP phosphorylation is part of a cooperative mechanism between axons and Schwann cells that together determine internode length, and suggest motor and sensory axons utilize different mechanisms to establish internode length.

## 1. Introduction

Postnatal development of peripheral nerves requires myelination and establishment of axonal diameter, which are required for rapid impulse transmission. Myelination includes formation of compact myelin and bidirectional elongation of myelinating Schwann cells along axons (Sherman and Brophy, 2005). While insights into the formation of compact myelin have been made, comparatively little is known about Schwann cell elongation. During development, there are two periods of Schwann cell elongation that establish internode length. The first period occurs during initial myelination from post-natal day 1 to approximately 14 days later, when myelination is complete (Webster, 1971). The initial period of elongation clusters ion channels (Dugandzija-Novakovic et al., 1995; Schafer et al., 2006; Vabnick et al., 1996) and establishes optimal internode length to maximize neuronal conduction velocity (Goldman and Albus, 1968; Hardy, 1971; Huxley and Stampfli, 1949). After initial establishment of internode length, Schwann cells continue to elongate during normal developmental growth to maintain optimal internode length and maximal conduction velocity (Bunge et al., 1989; Simpson et al., 2013; Vizoso, 1950).

A positive correlation between internode length and axon diameter was established over 140 years ago (Key and Retzius, 1875) such that larger diameter axons have longer internodes. Radial growth establishes axon diameter, and is dependent upon myelination (de Waegh et al., 1992). Myelination induces radial growth, so myelinated regions of an axon have larger diameters compared to unmyelinated regions of

*Abbreviations:* NF, neurofilament; NF-L, neurofilament light; NF-M, neurofilament medium; NF-H, Neurofilament heavy; KSP, Lysine-Serine-Proline; $Na_v$, Voltage gated sodium channel; EtBr, Ethidium bromide; MBP, Myelin basic protein; ROD, Relative optical density

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the same axon (nodes of Ranvier) (Yin et al., 1998). Increases in axonal diameter correlate with neurofilament (NF) number (Friede and Samorajski, 1970), which are the main cytoskeletal proteins of myelinated axons (Perrot et al., 2008). NFs are the intrinsic determinants of axonal diameter in myelinated axons (Eyer and Peterson, 1994; Ohara et al., 1993; Zhu et al., 1997). Furthermore, axonal neurofilaments are composed of three subunits namely NF-Light (NF-L), NF-Medium (NF-M), and NF-Heavy (NF-H), (Jones et al., 2016) of which NF-M determines axonal diameter (Elder et al., 1998; Garcia et al., 2003). Within myelinated axon regions, neurofilament heavy (NF-H) and medium (NF-M) become phosphorylated on C-terminal lysine-serine-proline (KSP) repeats (de Waegh et al., 1992; Julien and Mushynski, 1982) upon myelination (de Waegh et al., 1992; Yin et al., 1998). Moreover, NF composition varies with developmental stage (Shaw and Weber, 1982; Shen et al., 2010) and within central versus peripheral nervous systems (Yuan et al., 2006; Yuan et al., 2012). Thus, Schwann cell elongation, radial axonal growth, and NF KSP phosphorylation occur in parallel during postnatal development.

After nerve injury, the positive correlation between internode length and axon diameter is lost (Hildebrand et al., 1985; Vizoso and Young, 1948). Remyelination results in uniformly short internodes (Hildebrand et al., 1987; Sanders and Whitteridge, 1946; Vizoso and Young, 1948) and therefore reduced conduction velocity (Court et al., 2004). Loss of myelin also reverts several axonal properties to a pre-myelin phenotype. Sodium channels, normally clustered within nodes of Ranvier, become diffusely localized within the axonal plasma membrane after myelin loss (Boiko et al., 2001; Waxman et al., 2004). Demyelination results in re-expression of $Na_v1.2$ α subunit of voltage sensitive sodium channels (Craner et al., 2004; Schafer et al., 2006) and suppression of $Na_v1.6$ (Craner et al., 2004; Schafer et al., 2006), which is the predominant α subunit of myelinated peripheral nerves (Boiko et al., 2001; Schafer et al., 2006; Vabnick et al., 1996). However, one axonal property that does not revert to a pre-myelination phenotype is the phosphorylation status of NF proteins (Arroyo et al., 2004; Cole et al., 1994). In order for demyelinated axons to revert back and resemble developing axons before myelination (pre-myelination phenotype), both NF-M and NF-H would need to be dephosphorylated during demyelination. The significance of maintaining NF-M and NF-H phosphorylation during demyelination and after demyelination is currently unknown (Garcia et al., 2009).

In this study, we demonstrate that NF-M KSP phosphorylation is involved in regulating internode length during remyelination and myelination. Preventing NF-M KSP phosphorylation resulted in formation of longer internodes during remyelination. Mimicking constitutive NF-M KSP phosphorylation during normal myelination reduced internode length. These results provide the first mechanistic insights into reduced internode length that occurs with remyelination.

## 2. Materials and methods

### 2.1. Animals

Mice were housed in microisolator cages under a 12-h light/dark cycle and were given food and water ad libitum. All procedures were in compliance with the University of Missouri and University of California San Diego Animal Care and Use Committees and with all local and federal laws governing the humane treatment of animals.

### 2.2. Nerve demyelination by ethidium bromide injection

For demyelination experiments all mice used were 1 month old. Mice were placed under isoflurane anesthesia and the left sciatic nerve exposed via an incision in the flank followed by separation of underlying musculature by blunt dissection. Utilizing pulled microcapillary needles 2 μl of 0.1% ethidium bromide in normal saline was injected into the middle third of the exposed sciatic nerve to induce demyelination. Uninjected controls received only a sham operation, but no injection. Saline injected animals received an injection of normal saline. The mice were allowed to recover and placed back in their cage. Successful injection of 2 μl of 0.1% ethidium bromide into the sciatic nerve was confirmed by a change in colour of the nerve compared to the uninjected and saline injected controls. Seven days after injection, the animals injected with ethidium bromide displayed loss of function of the injected nerve, which is consistent with impaired nerve function after an injury (Villalon et al., 2015). Gross examination of the nerves at 7 days post-injection showed inflammation and discoloration of the ethidium bromide injected nerve (Supplementary Fig. 1). Sciatic nerves were collected for analysis at three different time points; maximal demyelination point, at 7 days post-injection; beginning of remyelination, 14 days post-injection; and complete remyelination point, 30 days post-injection (Bondan et al., 2009; Bondan and Monteiro Martins Mde, 2013; Riet-Correa et al., 2002). For detailed analysis of nerves after ethidium bromide injection, Swiss Webster mice were treated as described above. Seven days after injection, mice were killed by anesthetic overdose and decapitation and the sciatic nerve distal to the injection site removed into 2.5% glutaraldehyde before processing to resin blocks exactly as described elsewhere (Jones et al., 2016) and viewing via a light microscope.

### 2.3. Detection and quantification of NF proteins by immunoblotting

Sciatic nerves (demyelination experiments) and L5 motor and sensory roots were harvested and snap frozen in liquid nitrogen or were homogenized immediately. Tissues were 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 instructions. (Complete Mini, Roche, Mannheim, Germany). An equal volume of lysis buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 2% SDS was added. The homogenates were sonicated for 10 s 2×, then boiled for 10 min in a sand bath, 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, USA). Protein extracts (5 μg) were resolved on 7.5% SDS-polyacrylamide gels and transferred onto nitrocellulose membrane or stained with Coomassie-blue. NF-L, NF-M and NF-H were identified with polyclonal antibodies. Phosphorylated NF-H and NF-M were identified using a mouse monoclonal antibody that recognizes the protein in a phospho-dependent manner (SMI-31, Covance, Emeryville, CA, USA). Mouse and chicken primary antibodies were detected with donkey anti-mouse and goat anti-chicken secondary antibodies conjugated to IRdye-700X® infrared fluorophores (Rockland, Gilbertsville, PA, USA) respectively. Immunoreactive bands were visualized by infrared detection with an Odyssey image scanner (LICOR Biosciences, Lincoln, NE, USA). Quantification of immunoblots was performed by the relative optical density (ROD) method. Absolute intensities of the immunoreactive bands were obtained using Photoshop (Adobe Systems Inc.) and RODs were calculated in the following manner: [(NF protein mean intensity − background mean intensity) ∗ (number of pixels)]/[(loading control band mean intensity − background intensity) ∗ (number of pixels)]. As loading control the intensity of the smear on the coomassie stained gels was used as the stain intensity directly correlates with the amount of protein loaded in the gel. Optical densities of immunoreactive bands were calculated as follows: [(absolute intensity − background intensity) ∗ (number of pixels)] Average optical densities for each genotype were calculated and compared to the wild type averages, which were arbitrarily set to a value of 1. All optical densities were analyzed for statistical significance by one-way ANOVA with Holm-Sidak post hoc analysis for pair wise comparisons or by a Student's *t*-test (SigmaPlot, Systat Software).