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Distal to proximal development of peripheral nerves requires the expression of neurofilament heavy

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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

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 α-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

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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). Temporarily 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.

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 (4^{th}) 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 with1% 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.

Results

Radial axonal growth is reduced in proximal segments of motor axons from NF-$H^{-/-}$ mice

Previous analysis of motor axons in NF-$H^{-/-}$ 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^{th} lumbar ventral root, and distal, motor branch of the peroneal nerve, segments of the sciatic nerve (Figure 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 1A). The peak diameter of the largest fibers is reduced in NF-$H^{-/-}$ lumbar roots relative to wild type control (Figure 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-$H^{-/-}$ proximal nerve segments (Figure 1B). Moreover, two other previous studies of NF-$H^{-/-}$ 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-$H^{-/-}$ mice (Figure 1C). There was no difference in the peak diameter obtained by wild type or NF-$H^{-/-}$ mice (Figure 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-$H^{-/-}$ axons contribute 18% to the total number of axons within the range of 6-9μm whereas NF-$H^{-/-}$ axons contribute 35% to the total population of axons within the range of 2-8μm 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 1D).

These results suggest that deletion of the NF-H subunit has a greater affect on peak diameter distribution and 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 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 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 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 2D). However, diameter distributions for NF-H$^{-/-}$ mice start to become smaller in segments closer to the spinal cord. In segments 2 and 3, the peak diameter is reduced by .5 μm in NF-H$^{-/-}$ mice (Figure 2E and F). In the most proximal segment, segment 4, peak diameter in NF-H$^{-/-}$ mice is reduced 1.5 μm relative to wild type mice (Figure 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 .5 μm as axons approach their target (Figure 2D and G). However, axons in NF-H$^{-/-}$ mice are the smallest as they exist the spinal cord (Figure 2G) increasing as they approach their targets. NF-H$^{-/-}$ 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$^{-/-}$ 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$^{-/-}$ 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 3A). NF spacing was determined for segments 1 and 4 in both wild type and NF-H$^{-/-}$ axons. For segment 1, the peak in nearest neighbor distances was similar in wild type versus NF-H$^{-/-}$ axons (Figure 3A). However, NF-H$^{-/-}$ axons appear to have more NFs that are spaced at and below 27 nm whereas wild type axons have more NFs that are spaced between 34-51 nm (Figure 3A). Moreover, there were no differences in the total number of NFs in wild type versus NF-H$^{-/-}$ in segment 1 (Figure 3B). Analysis of NF spacing in segment 4 resulted in a much different profile than segment 1 (Figure 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 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 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 3A upper versus lower panel). This difference in spacing was also noted in NF-H$^{-/-}$ axons (Figure 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.

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 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 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 3C and D), as NF content is a major determinant of axonal diameter in myelinated fibers (Friede and Samorajski, 1970; Hoffman et al., 1984; Perrot et al., 2008; 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.

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Abbreviations List

E_	Embryonic day _
NF	Neurofilament
NF-H	Neurofilament Heavy
NF-L	Neurofilament Light
NF-M	Neurofilament Medium

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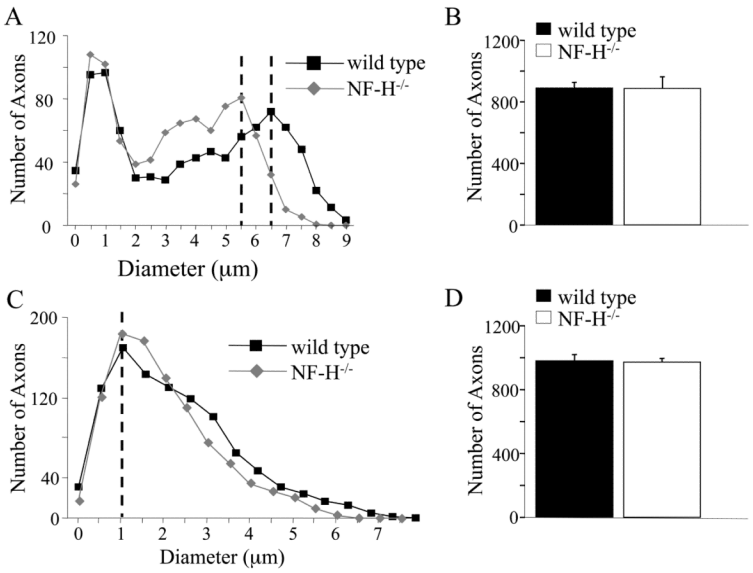

Figure 1. Radial growth of proximal axonal segments is most affected by loss of NF-H in sciatic nerve

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 and age group. 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.

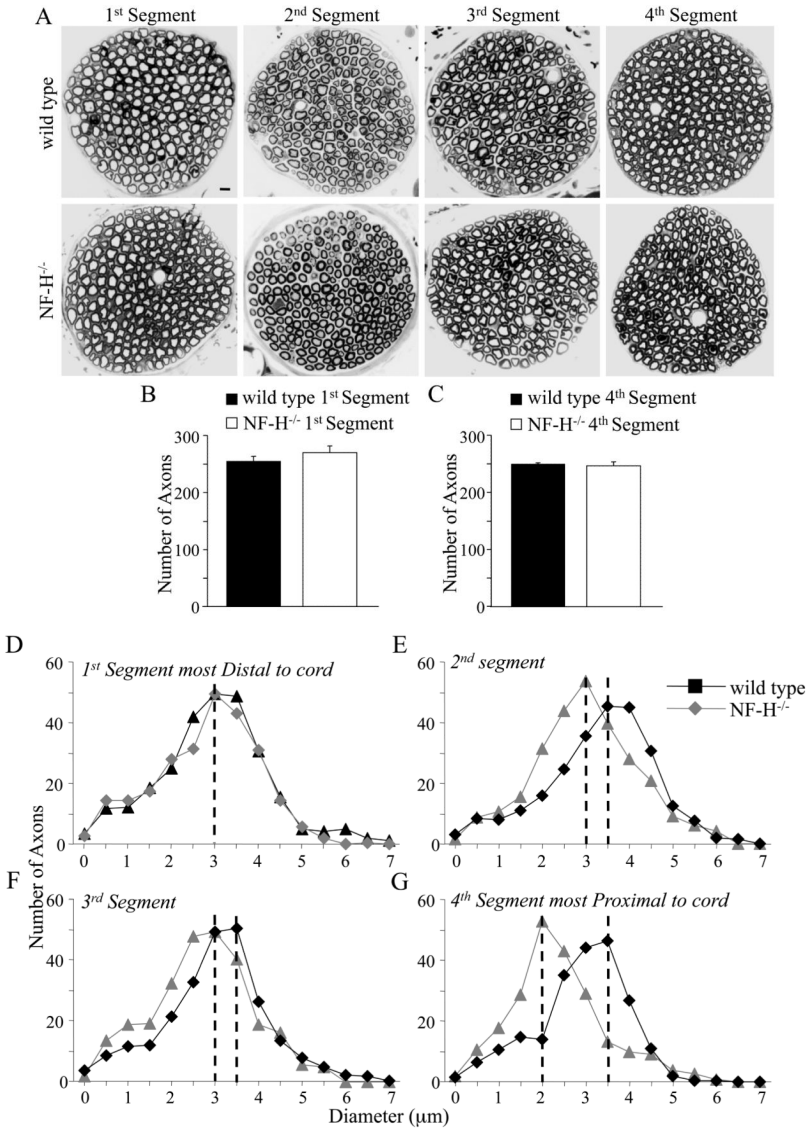

Figure 2. Reductions in axonal diameter occur along the length of the phrenic nerve

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. Bar, 5μm. (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. (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).

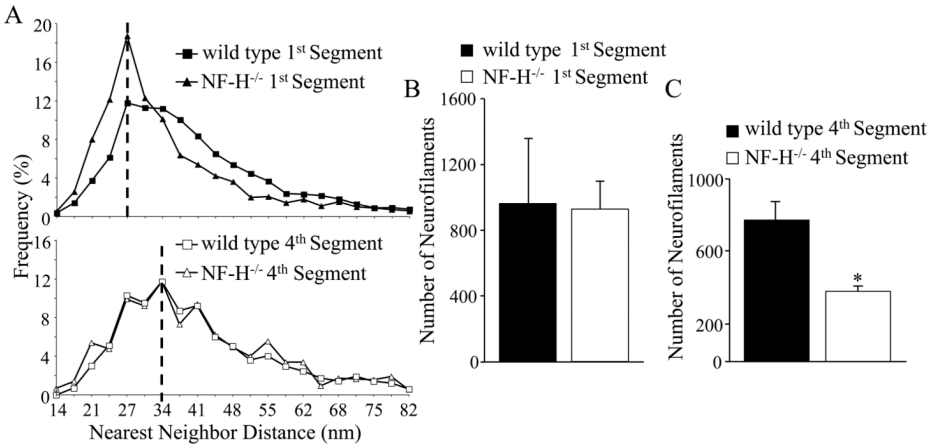

Figure 3. Neurofilament nearest neighbor distances are larger in distal axonal segments in both wild type and NF-H$^{-/-}$ axons

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$^{-/-}$ 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$^{-/-}$ mice. Neurofilament number is significantly reduced in Segment 4 of NF-H$^{-/-}$ axons relative to wild type Segment 4 and NF-H$^{-/-}$ Segment 1. Neurofilament numbers were analyzed for statistical significance using unpaired Student's *t*-test. *, P value < 0.05.