A Morphometric Study of Nerve Fiber Atrophy in Rat Spinal Roots

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Abstract. A morphometric study of atrophy of nerve fibers was made in the ventral and dorsal roots of rats, four to 48 weeks (wk) after transection of their sciatic nerves and with regeneration prevented. The pathophysiological events of fiber atrophy may be summarized as follows: wasting of the axon caliber coincides with a loss of neurofilaments with relative preservation of microtubules. This leads to non-circularity of the fiber, evident from four wk on. The caliber of the circular profile is also reduced. Adaptive changes in myelin sheath structure follow. Thinner fibers with relatively thick sheaths are first detectable after eight wk and prominent after 24 to 48 wk. This change may indicate passive slippage of the sheath, but sheath remodelling in adjustment to a changed internodal geometry appears more likely.

Key Words: Atrophy, nerve fiber; Myelin sheath, thickness; Non-circularity, nerve fiber; Peripheral nerves; Spinal nerve roots.

INTRODUCTION

The experimental induction of atrophy of peripheral nerve fibers requires a severe and lasting disturbance of the neuron's economy. A reduced functional input does not suffice. There is no atrophy of ventral roots after rhizotomy and cord hemisection (1), nor are there consistent changes after tenectomy (2–4) or after limb immobilization (5, 6). Local compression of adult nerves may cause distal atrophy (7, 8), whereas compression during growth causes distal hypoplasia (9–11). Atrophy from compression is clinically relevant, e.g., to the carpal tunnel syndrome (12), but the severity of changes may vary across the profile of the nerve from loss of superficial fibers to slight change in the center of the nerve.

Another widely used experimental model is the atrophy of fibers in the proximal stumps of transected nerves in which regeneration was prevented (13–20). Gillespie and Stein (21) made a comprehensive morphometric analysis of the proportions of the myelin sheath of such fibers.

One objective criterion for defining atrophy is to establish that the internodes of a fiber are too thin for their length (7). This is because atrophy may reduce fiber caliber, but it cannot reduce internode length unless there is additional demyelination and remyelination. The changes developing in the profiles of such atrophic fibers are more difficult to interpret. Small, nearly circular axon profiles with exceptionally thick myelin sheaths were thought to indicate atrophy (8, 22). On the other hand, atrophic nerves also show collapse of the axon's profile (17) with increased non-circularity (7, 20, 21, 23). The present study was directed at a better understanding of the significance of these phenomena. We studied spinal roots (17) rather than...
nerve trunks; this reduces the scatter in the data, since atrophy does not affect afferent and efferent fibers alike (18, 19, 24). We also used a morphometric program (25) which provides several options for analysing fiber structure, and we included data on the changes in the axonal cytoskeleton (23, 26).

MATERIALS AND METHODS

Young adult Sprague-Dawley rats weighing 200 to 250 g at the time of surgery were used. The left sciatic nerve was transected 1–2 mm distal to the origin of its branch to the gluteus superficialis and a nerve segment of 10–15 mm length was excised. The proximal and distal stumps were tightly ligated with 6/0 Prolene®; the distal stumps were reversed and sewn to the skin to prevent functional regeneration. The animals were killed 4, 8, 24 and 48 wk after surgery. There were three animals for each group, four for the eight-wk survival. The four-wk survivors weighed 300 to 420 g. After 24 to 48 wk the animals had attained weights of 400 to 500 g, except for one animal in the 48-wk group which weighed 660 g. Muscular atrophy of the lower leg was marked after eight wk. The prevention of regeneration was verified when each animal was killed. There was no twitching of the muscles of the lower leg when the neuroma was excised and there was no outgrowth of regenerating nerve fascicles to the lower leg or to the gluteus superficialis muscle.

The roots were prefixed in situ by injecting glutaraldehyde into the subarachnoid space immediately after decapitation of the deeply anesthetized animals. The spinal roots were dissected in continuity with the plexus ischiadicus to ascertain identification of the lumbar segments. The distal half (between cord and ganglia) of the root pair L5 was used for measurements. Contrary to L4, it did not receive fascicles from the gracilis and femoralis nerves. Short segments of the identified roots were postfixed for two hours in 2.5% glutaraldehyde in 0.2% cacodylate buffer, osmicated and embedded in Araldite. Thin sections were stained with uranyl acetate and lead citrate and were examined with a Zeiss EM10 electron microscope.

For morphometry, electron micrographs were taken at ×1,300 of every grid opening to provide random sampling. Both inner and outer circumferences of the myelin sheaths were traced manually with a cursor, using a Kontron videoplan. Special computer software, developed to our design by the Kontron company, determined the circumferences of the axis cylinder and of the outer surface of the sheath and the areas of axoplasm and of myelin. From these primary parameters, six secondary parameters were calculated including the axon and fiber diameters for a full circle based on circumference, the area of the circular axon based on circumference, the thickness of the myelin sheath, the g-ratio (quotient axon diameter/fiber diameter) and the non-circularity factor (measured area of axoplasm/area of circle of the same circumference). There is a choice of parameter combinations; this is of advantage for studying the relationship between circular fiber caliber and the superimposed changes from non-circularity.

To study subpopulations, one may use a computer window restricting the abscissa to a narrowed range, e.g. fibers of 0 to 2 μm caliber. A 20 × 20 count matrix, printed for each of the scatter diagrams, was used for additional cluster analysis. This count matrix is routinely supplied by the Kontron program. It looks like the scatter diagrams in Figure 6, except that numbers are printed showing densities of points. Small windows can be delineated in this data matrix and the frequency of a specific subpopulation can be expressed in per mill of total counts. The shift of subpopulation densities may be shown in this way.

For counts of neurofilament and microtubule densities, it was uncommon to have fibers with all their neurofilaments cut exactly perpendicular to axis. To avoid ambiguities in counting, areas of axoplasm in which all filaments and tubules were cut exactly perpendicular to their axis were delineated. These areas excluded mitochondria and those filaments or tubules lying in their immediate proximity. A square grid was then superimposed at random and filaments and tubules were counted in each square that lay completely within the delineated area. The total number of filaments and tubules was then calculated for the measured area of axoplasm, or for the circular axon profile, respectively.

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RESULTS

Light and Electron Microscopic Observations

Light microscopic examination of semithin sections of ventral roots showed no recognizable difference between the left root (side of transection) and the right root. The dorsal roots, in contrast, showed obvious differences from eight wk on. The majority of the fibers of the left root were collapsed, with narrow, slit-shaped or irregular axon profiles having no preferential orientation of their long axes (Fig. 1). There were always a few uncollapsed, rounded profiles between these fibers. These were thought to be fibers stemming from the unsevered branches to the lumbar and the superficial gluteal muscles. There were no signs of fiber loss, of degenerating fiber profiles or of myelin figures. The fibers of the right roots showed no signs of collapse; they had their normal cogwheel profiles. In addition to the collapse of fibers in the left roots there was also an abnormal population of fibers of thin caliber with abnormally thin myelin sheaths. These increased with time, becoming quite numerous with longer survival. They were thought to represent a newly formed population of regenerating fibers. No such fibers were seen in the right dorsal root or in the ventral roots.

The difference between the shrunken dorsal root fibers and the unaltered ventral root fibers allowed one to trace the dorsal root fascicles across the fusion of the two roots by using additional tissue samples of the ganglia and distal to them. A proximo-distal step series of semithin sections showed the lenticular fascicles of shrunken dorsal root fibers interwoven with the ventral root fibers. This pattern was taken as evidence that the shrinkage of the dorsal root fibers was a true change and not one produced by conditions of tissue processing (Fig. 1).
Fig. 2. Histograms of non-circularity factors show no obvious difference between the left and the right dorsal root after four wk (compare text). After eight to 48 wk, there is a marked decrease in non-circularity factors in the fibers of the left root. The right-side controls for these survival periods are not shown; they were congruent with the four-wk sample.

Electron micrographs showed the changes in fiber configuration seen in the semithin sections. The periodicity of the myelin lamellae was unchanged in the collapsed fibers. A few fibers had relatively thin axons encompassed by very thick myelin sheaths. Such fibers were usually rounded and lacked the irregular configuration seen in the collapsed population.

Morphometric Data: General Remarks

Three animals were measured for each survival period with the exception of four from the eight-wk period. The data were similar within each group, and they are shown pooled here. For the right dorsal roots, serving as controls, a total of 4,521 fibers was measured. For the left dorsal roots we measured 1,210 fibers for the four-wk survivors, 2,051 fibers for the eight-wk, 932 fibers for the 24-wk and 1,581 fibers for the 48-wk.

A parallel set was obtained for the ventral roots, after 4-, 24- and 48-wk survival. A total of 1,342 fibers was measured for the right ventral roots, 1,544 for the left ones. These data are not shown here, as no significant differences were found between left and right ventral roots.

Non-circularity of Fibers

The non-circularity factor (area axon measured/area of a circle of the same circumference) may be printed in frequency histograms (Fig. 2) or in scatter diagrams versus fiber caliber (Fig. 3). Non-circularity factors for the right control roots averaged between 0.58 and 0.63. This was low compared to the values near 0.7 obtained with the same technique in mammalian sciatic nerves (25). The difference was attributed to tissue processing and also to the tighter packing of root fibers.

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Fig. 3. Scatter diagrams of non-circularity factors after 24-wk survival show the extent of axon shrinkage for different calibers. The diagram on the right side shows the shrinkage of fibers in the left root; the left diagram shows the corresponding right root.

The left spinal roots had distinctly lower non-circularity factors than the right roots and their frequency histograms showed a definite shift to the left (Fig. 2). Differences between left and right dorsal roots were obvious for 8, 24 and 48 wk of survival (Fig. 2). After four wk of survival, the difference was not as obvious on inspection of the histograms. A Kolmogoroff Smirnov test showed a significant difference ($\chi^2$: 52), but this was mainly caused by one of the three animals ($\chi^2$: 1.72; 6.12; 107.6).

Changes in the Axonal Cytoskeleton

Counts of microtubule and neurofilament densities were made in 19 fibers from the control right dorsal roots and in 15 fibers from the atrophic left dorsal roots after 24-wk survival. The latter were selected for non-circularity (factors below 0.5), or relatively thick sheaths (g-ratios below 0.5). The range of fiber calibers was similar in the two groups.

The measured neurofilament density multiplied by the axon area yields the total number of filaments per axon which is plotted against axon area corrected for circularity (Fig. 4). The regression was significantly less steep in atrophic fibers ($t$: 8.41; significance level 1%). There were fewer neurofilaments in the atrophic fibers than would correspond to circular profiles. Hence, increased non-circularity was not from simple shrinkage or dehyration of normal axons; rather, there was a wasting of the axon's cytoskeleton.

The numbers of microtubules per circular axon had greater scatter around the regression line, but there was no difference between atrophic fibers and controls. This shows that the reduction in the number of neurofilaments during atrophy was not accompanied by similar changes in the number of microtubules (Fig. 5), which remained constant.

A statistical analysis of neurofilament and microtubule densities confirmed these observations (Table 1). The density of microtubules was significantly increased after atrophy. However, when the densities were corrected for circular profiles (density $\times$ non-circularity factor), no difference was seen. This means that the total number of microtubules did not change with collapse. Almost the reverse was found for the neurofilaments; their density corrected for circular profiles was significantly reduced after atrophy. There was a less significant increase in neurofilament density (not corrected for circularity). Hence, the reduction in neurofilaments did not quite keep pace with the collapse of the axis cylinder.
Fig. 4. Counts of neurofilaments and microtubules in fibers of the right and the left dorsal root show greatly reduced numbers of neurofilaments per circular axon profile in the left root. There is no corresponding reduction in the number of microtubules per circular axon profile. Non-circularity, therefore, corresponds to a selective loss of neurofilaments. Note the difference in scales.

Myelin Sheath Thickness

The most informative scatter diagrams were of the g-ratio (quotient axon diameter/fiber diameter) plotted against circular axon diameter (Fig. 6). The normal distribution of the g-ratio in the right (control) dorsal roots showed a characteristic pattern known from a study of peripheral nerves in several species (25). For the majority of fibers there was an increase in the g-ratio (decrease in sheath thickness) with fiber caliber, resulting in a linear regression of g. The very thin fibers at the left end of the scatter diagram formed a cluster, having relatively thin sheaths (high g-ratio). These fibers are known to form a separate population in some species, e.g. in the frog and the cat (25). This is of interest as the type of change seen in atrophic roots was quite different for these thin fibers and for the rest of the fiber population.

The population of thin fibers at the left end of the scatter diagram was transformed into (or replaced by) a population of fibers having still thinner sheaths. With light microscopic inspection these were the fibers thought to be degenerates. The transformation of this population could be shown by using a computer window for fibers of 0 to 2 μm caliber. Their mean g-ratio was 0.59 at four wk, 0.60 at eight wk, 0.62 at 24 wk and 0.64 at 48 wk, but the statistical significance of these changes was uncertain. A more accurate insight into the increase of these fibers was obtained by using a smaller computer window restricted to calibers between 1.5 and 3.0 μm and g-ratios between 0.75 and 0.9. For this subpopulation, the normal right roots gave between 2 and 19 per mil. In the left roots there were changes from 7 to 30 to 66 to 111%, showing a steady increase in the frequency of this subpopulation. The origin of these fibers was not clear. We found no evidence to support the assumption that they were retrograde, ascending degenerates from the nerve stumps. When roots were studied in three animals one wk after excision of the neuroma at the stump, there was no degeneration of these thin fibers.

The changes observed in the fibers of medium to large caliber were entirely different. There was a progressive rarefaction of fibers at the right end of the scatter diagram (Fig. 6). This indicated axon atrophy even for circular axon profiles. Corresponding to this rarefaction of fibers, there was an increased density of fibers of medium caliber. After 24 to 48 wk of survival, a new fiber population had formed at the left lower end of the regression line and had calibers between 2 and 5 μm and
Fig. 5. Neurofilament density in a fiber of the non-atrophic right dorsal root is shown in the left picture; there are relatively few microtubules. The right picture shows an atrophic fiber from the left root. The axon is collapsed to form a U-shaped slit. Neurofilament density is normal, but the number of microtubules is markedly increased. × 46,980.
TABLE 1

Densities of Neurofilaments and Microtubules in Normal and Atrophic Nerve Fibers

<table>
<thead>
<tr>
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<th>Mean</th>
<th>Standard deviation</th>
<th>Degrees of freedom</th>
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<tr>
<td>Neurofilaments/μm² axoplasm:</td>
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<tr>
<td>Right dorsal root</td>
<td>273</td>
<td>58</td>
<td>17</td>
<td>2.71*</td>
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<tr>
<td>Left dorsal root</td>
<td>383</td>
<td>148</td>
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<tr>
<td>Neurofilaments/μm² axoplasm, corrected for circularity:</td>
<td></td>
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<tr>
<td>Right dorsal root</td>
<td>198</td>
<td>37.7</td>
<td></td>
<td>4.16†</td>
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<tr>
<td>Left dorsal root</td>
<td>112</td>
<td>72.6</td>
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<td>Microtubules/μm² axoplasm:</td>
<td></td>
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<tr>
<td>Right dorsal root</td>
<td>32.4</td>
<td>10.8</td>
<td>17</td>
<td>5.67†</td>
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<tr>
<td>Left dorsal root</td>
<td>87.9</td>
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<td>Microtubules/μm² axoplasm, corrected for circularity:</td>
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<td>9.6</td>
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<tr>
<td>Left dorsal root</td>
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<td>23.4</td>
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* Significance level: 5%.
† Significance level: 1%.

very low g-ratios of 0.4 to 0.5. Normal roots had no such fibers with very thick sheaths. The scatter diagrams, accordingly, indicated a change which had caused the plots for the fibers to shift toward the left and down the regression line. The redistribution of fibers along the regression line could be demonstrated by the use of two small computer windows: one for fibers of 6 to 7.5 μm caliber and 0.6 to 0.7 g-ratio, characterizing the fibers of medium caliber; one for fibers of 2.25 to 3.75 μm caliber and a g-ratio of 0.6 to 0.7, characterizing the “new” population of fibers with relatively thick sheaths. The former were found to increase from 7 to 24 to 178 to 44% (controls between 1 and 15%), while the latter decreased from 93 to 48 to 39 to 37% (normal values 90 to 125%). These data confirm the impression, obtained from the inspection of the scatter diagrams, that there was a general shift to the left, or to thinner caliber ranges with an increase in sheath thickness, respectively. They also document that the increased non-circularity found early during atrophy was accompanied by an early decrease in the circumference of the circular profiles of the fiber.

DISCUSSION

The difference between ventral and dorsal roots found here agrees with the difference in the reduction of conduction velocities in dorsal and ventral root fibers (18, 24, 27, 28). The collapse of axons explains the reduced conduction velocity, similar to IDPN neuropathy (29). If the cross-sectional area of axons is reduced osmotically in vitro, there is a corresponding reduction in conduction velocity, and both changes are reversible upon restoring normal osmolarity (30). The absence of ventral root changes in our material disagrees with the data of Hoffman et al (23); however, their findings were for young rats, and the changes were most marked in the proximal root segment. The different responses to axotomy in dorsal and ventral roots may correspond to the degree of residual activity in those fibers. Dorsal root fibers are completely deafferented, while the stumps of ventral root fibers are not.
Fig. 6. Changes in myelin sheath thickness of left and right roots after 24- and 48-wk survival. The scatter diagrams show the g-ratio (quotient axon diameter/fiber diameter) for circular fiber profiles. The location of the computer window used for cluster analysis is indicated. The upper window serves as a reference for the regenerating fibers of thin caliber which have very thin sheaths. The two lower windows display the shift in density to the left side of the scatter diagrams, and the corresponding appearance of a “new” group of thin fibers with abnormally thick sheaths.

Atrophy of a peripheral nerve fiber may, in principle, involve several types of interaction between the axon and the Schwann cell in the fiber's profile: A. The axon and myelin sheath may waste concurrently, leading to a thinner fiber of essentially normal proportions. B. The sheath may waste faster than the axis cylinder, leading to “hypomyelination.” C. Axon wasting may precede sheath wasting, which may lead to collapse of the axis cylinder with increased non-circularity or rearrangement of the sheath; the volume of myelin per axon caliber will increase.

The second possibility is not supported by our data. The assumption that the fibers of thin caliber with thin myelin sheaths are atrophic must be rejected. These fibers had the morphometric attributes of sprouting, regenerating fibers (31). Their origin remains obscure. Sprouting fibers may ascend from the nerve stumps (32, 33). However, the persistence of the fibers in the roots after excision of the neuroma speaks against an origin from the nerve stumps. More likely, the changes in roots are akin to a neuropathy (34), which agrees with the occurrence of nodal lengthening and internodal demyelination in atrophic fibers (20).

The two other possibilities of axon sheath interaction mentioned above are not mutually exclusive. The most striking changes in the atrophic dorsal root fibers were their collapse and decreased non-circularity factor, consistent with the data of Dyck et al (20) and Gillespie and Stein (21). This also corresponds to a reduced area of axoplasm relative to myelin area (7). However, the non-circularity factor measured
in a tissue section is the complex product of the in vivo state of the fiber (35, 36), of the osmolarity of body fluids (37) and of the composition of the media used for fixation and processing (30, 38, 39). The degree of non-circularity caused by atrophy is the difference between the change attributable to atrophy and the sum of all other factors. This difference was verified statistically in the present material, and it was also evident from the different behavior of dorsal and ventral root fibers lying adjacent in the same section.

The shrinkage of the axis cylinder of atrophic fibers could result from the wasting of substance, or from a simple compaction due to dehydration with increased density of axoplasm. The distinction can be made from the axon's cytoskeleton (40–43). Shrinkage from osmotic dehydration of fibers causes increases in the density of neurofilaments (44). Conversely, if filament density does not increase in the collapsed fiber profiles, this shows wasting of the axonal cytoskeleton. Our counts showed that the total number of neurofilaments was significantly less than would correspond to the circular profiles of non-atrophic fibers. Rather, their number was adapted to the reduced axon area. In terms of microtubules, there was no change in total number, compared with circular fiber profiles. Axon wasting, therefore, was from a preferential loss of neurofilaments with preservation of microtubules similar to that in hypoplastic fibers distal to a compression (23, 45) or in neuropathy (26). This observation is consistent with the proposal that axon caliber is determined mainly by the number of neurofilaments (44, 46–49). Atrophy correlates with the loss of neurofilaments in the axon's cytoskeleton (45).

Wasting of the axon's cytoskeleton and collapse of the axis cylinder results in increased non-circularity of the fiber (20, 21, 23). This change was manifest after four to eight wk. There was also a reduction in the axon's circumference, evident upon correction of axon caliber for circular profiles. Consequently, the thick fibers shift toward the smaller caliber ranges (20). This shift could also result from a preferential loss of thick fibers. No such preferential loss was found by Ygge and Aldskogius (50). Also, selective loss of thick fibers could explain fewer fibers at the right end of the scatter diagram, but it could not explain the appearance of a “new” population (thin fibers with thick sheaths) at the lower left end (Fig. 6).

If myelin sheaths did not respond to axon wasting at all, the only change would be a decrease in the non-circularity factor, and scatter diagrams of the g-ratio would be unchanged, provided they are plotted for circular fiber diameters. If axon atrophy is followed by wasting of the sheath, the resultant changes are less conspicuous, as such fibers simply shift to the left side of the scatter diagram.

The scatter diagrams of the g-ratios show a third phenomenon. In the later stages of atrophy, after 24 and 48 wk, a “new” population of fibers with very thick sheaths (g-ratio 0.4 to 0.5) had formed at the 2- to 5-μm caliber range. Such fibers may result from passive inward slippage of their sheaths, or from remodelling of sheath structure.

Outward slippage of myelin sheaths develops rapidly in the swollen stumps of severed fibers, and the number of turns is reduced in exact proportion to the increase in circumference (51). Conversely, an increased thickness of myelin sheaths during early Wallerian degeneration was interpreted inward slippage (52). If nerves are kept in millipore chambers which do not admit migratory phagocytes, the myelin sheaths are rejected by the Schwann cells. Such rejected sheaths lying free from Schwann cells shrink upon the central lumen, with a corresponding increase in thickness, in a way which can be explained only by inward slippage (53). However, this type of
sheath slippage has so far been documented only when there are disruptive lesions. A more complicated remodelling was shown by O’Neill et al (8) from marked variation in axon caliber, but constant numbers of myelin turns per internode. An alternate aspect of sheath adjustment needs to be considered: the thickness of myelin sheaths does not only increase with axon caliber; the relative length of the internode is also important. Very long internodes have slightly thicker sheaths than short internodes of the same caliber (31, 34). This means that the l/d quotient (length/diameter) affects sheath thickness. The disproportionally thick sheath of atrophic fibers, therefore, may simply be an adaptation to the increase in the quotient internode length/axon diameter. This adaptation would be opposite to that found for presumably “hypomyelinated” Dystrophic, Quaking and Trembler mutants in which the abnormally thin sheaths corresponded to the degree of foreshortening of their abnormal internode populations (55). The absence of disruptive changes, and the very late development of increased sheath thickness in our material would speak for this type of sheath adjustment.

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