Journal of Neurocytology **20**, 450–458 (1991)

Axonal cytoskeleton at the nodes of Ranvier

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Received 12 June 1990; revised 5 October and 12 November 1990; accepted 23 November 1990

Summary

The relationship between the degree of nodal narrowing and the changes in the structure of the axonal cytoskeleton was studied in 53 fibres of mouse sciatic nerve. Nodal narrowing increased with increasing fibre calibre to reach about 20% of the internodal area in the thicker fibres. The narrowing corresponded quantitatively to a decreased number of nodal neurofilaments. Nodal microtubule numbers varied greatly, and a majority of fibres had considerably (approximately 55%) more microtubules in their nodal profile than in the internode. Nodal profiles of different calibre showed an increase in the number of filaments and of microtubules with nodal calibre, although at rates different from those in the internode. The degree of observed axon non-circularities had no discernible effect on the restructuring of the axonal cytoskeleton at the node. A transnodal transport of the axonal cytoskeleton can occur with: (1) accelerated transnodal transport of filaments, (2) stationary internodal fraction of filaments, (3) depolymerization of filaments proximal to the node and repolymerization distally, or (4) different nodal and internodal polymerization equilibria.

Introduction

This investigation concerns the relationship between reduction in the calibre of the axon at nodes (Rydmark, 1981; Rydmark & Berthold, 1983) and the corresponding changes in the structure of the axonal cytoskeleton. Berthold's (1968, 1978) comprehensive inventory of nodes and internodes from serial sections of six fibres provided such data. In the present study we used an approach similar to that of Rydmark (1981) and of Price and co-workers (1988a), identifying nodal profiles in the nerve and then tracing these fibres in serial sections along the nerve to obtain corresponding internodal profiles. The data define the extent to which restructuring of the nodal cytoskeleton occurs in fibres of different calibre.

Materials and methods

Three young adult mice (14 weeks) of C57 black strain, weighing 22–25 g, were used. They were perfused, under deep anaesthesia with Rompun-Ketanest, from the ascending aorta with 1% glutaraldehyde in PBS buffer at room temperature. The left sciatic nerve was removed, postfixed overnight at 4° C in 3% glutaraldehyde, osmicated in 2% osmium tetroxide and embedded in Araldite.

A complete series of approximately 1350 thin sections (pale silver, approximately 800 Å) were cut in each nerve. Each series was divided into three parts: the first part served for the collection of nodal profiles; the second part served for the tracing of fibres for a constant distance between nodal and internodal profiles; the third part served for the collection of internodal profiles. At the start of each series, a section was mounted on a Formvar-coated slit grid (in which no part of the nerve's profile is obscured) and a photographic reconstruction of the entire nerve profile was assembled from fields taken at ×1300. Each myelinated axon received a number; there were 1643 axons in the first animal, 1567 in the second and 1547 in the third. A calibration grid was photographed at the beginning of each electron microscopy session.

The first part of the series consisted of 450 sections; every third section was mounted on net grids. Every group of four net grids was followed by one Formvar-coated slit grid for survey. Only nodal profiles, encompassed by a collar of microvilli, without myelin and cut perpendicular to the axis of microtubules and neurofilaments, were selected. They were photographed at magnifications of 8000 and 80 000.

Additional survey montages were assembled from the Formvar grids, 100 sections apart, in the manner described earlier. The numbering of axons was repeated in each montage.

The second part of each series consisted of 600 sections of which every 20th was mounted on Formvar grids to continue the tracing of fibres in the manner described.

The third part of each series consisted of 300 sections; every fifth section was mounted on net grids and each group of four net grids was followed by one Formvar-coated grid,

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with a survey montage in which the numbered fibres were identified. Internodal profiles were photographed at ×8000 and 80 000; photomontages of the fibre profiles were assembled. Fibres were then traced back to the nodes to double-check identity.

The three parts of each series traced the entire myelinated fibre population of the nerve samples. We obtained 17 to 18 sets of nodal and internodal profiles per animal. The distance between them was 1100 ± 20 sections. No allowance was made for the different length of the internodes of thin and thick fibres.

Neurofilaments and microtubules were counted at a final magnification of ×330 000. A plastic foil was placed on the montages, and each counted neurofilament or microtubule was marked. Measurements were made with a Kontron videoplan at a magnification of 1300. The programme measures areas and circumferences, calculates non-circularity and corrects data for circular profiles based on either area or on circumference (Friede & Beuche, 1985).

Some technical problems may be considered here. The number of neurofilaments in the thicker fibres was probably slightly underestimated owing to distortions of fibre geometry from chemical fixation. Axons will appear too large and sheaths too thin if sheath shrinkage (the shrinkage of interperiod width) is greater than tissue shrinkage (Berthold *et al.*, 1982; Friede, 1986). This effect is rather small for thin fibres, but becomes considerable for thick fibres. Our sample, however, consisted mostly of thin fibres, and the error from sheath shrinkage was minor.

Clefts of Schmidt–Lanterman were excluded in principle because it is known that they can widen *in vivo* very rapidly within 2 min (Williams & Hall, 1971), causing rapid changes in the enclosed axon.

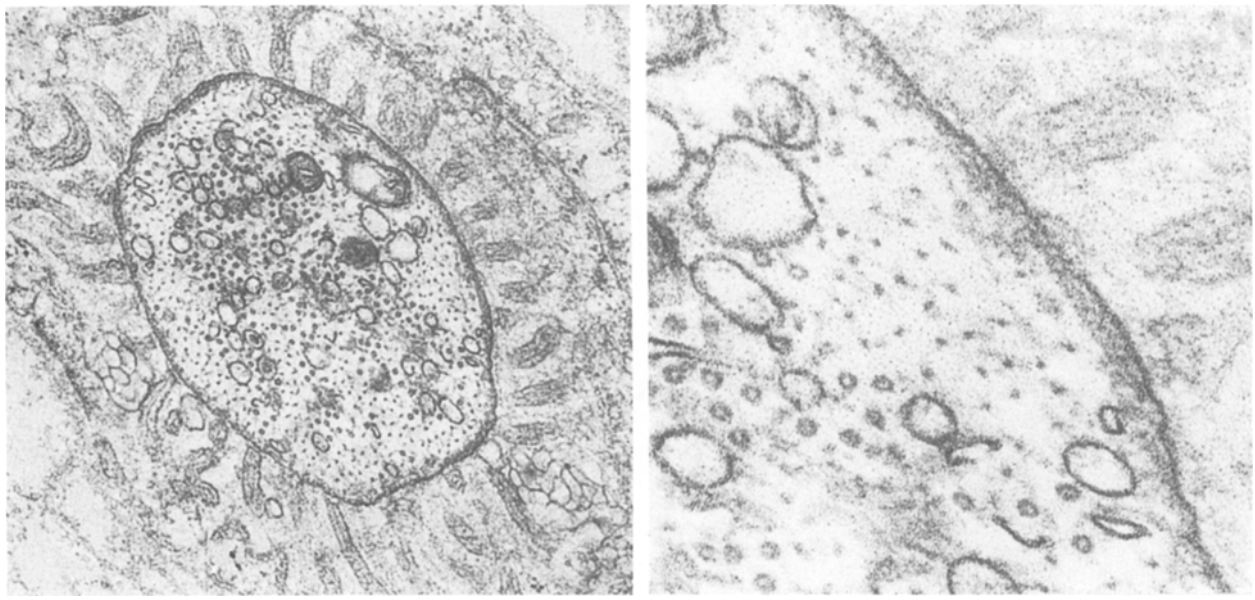
Morphometric studies of the axonal cytoskeleton should be based on total filament (or microtubule) numbers and on fibre non-circularity rather than on density values. Chemical fixation *per se* (superimposed by osmotic effects) shrinks axons, reducing non-circularity factors from 0.91 (in freeze-substituted specimens) to 0.68 (Minwegen & Friede, 1984). An atrophic fibre, for instance, may show increased filament density in spite of an actual loss of filaments if there is more shrinkage (non-circularity) than corresponds to filament loss (Friede *et al.*, 1985).

Single measurements per node do not account for the height of the bulge of the nodal membrane (Rydmark & Berthold, 1983). This variation was minimized by collecting only the short microvilli-containing domains of the nodal gaps which are immediately adjacent to the narrowest regions of the axon.

Results

Database and representativeness

Our database consists of 53 sets of nodal and corresponding internodal profiles from three animals, with equal numbers for each animal (Fig. 1). Data were



	Node	*Corresponding internode*
Axon diameter, circular	1.70	2.25
Axon area, circular	2.28	3.97
Noncircularity factor	0.70	0.82
g-ratio	–	0.60
Number of filaments	361	1005
Number of microtubules	152	116

Fig. 1. Nodal profile (× 34 970) and high power field (× 146 880) is shown along with an inventory of some critical parameters for the nodal profile and for its corresponding internode.

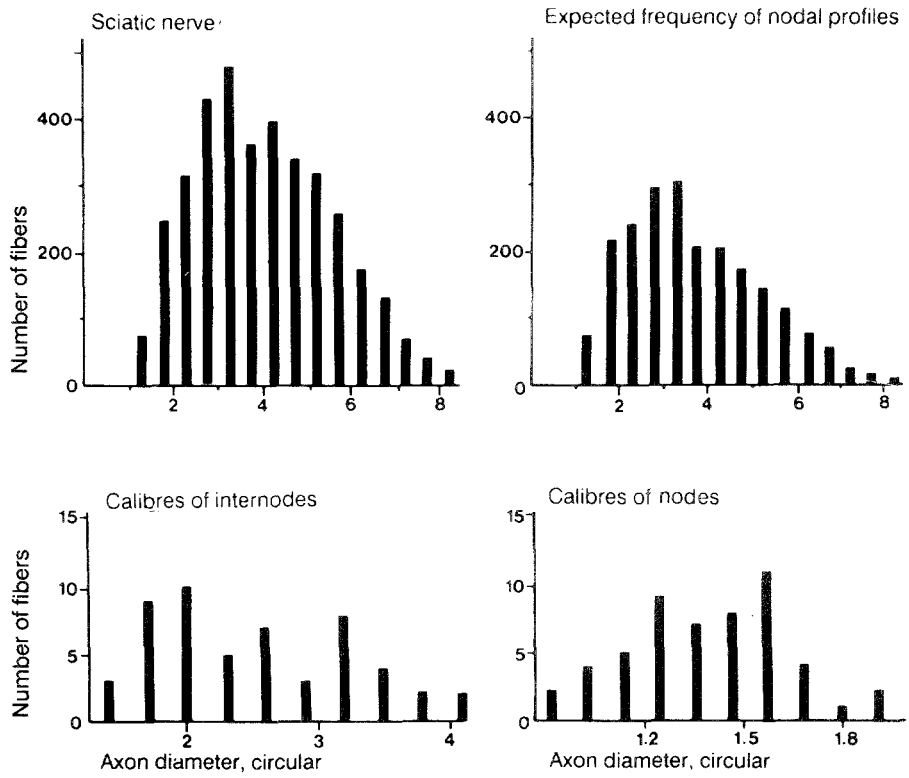


Fig. 2. Histograms of axon calibres. Top left: 3661 fibres representing the population of the sciatic nerve. Top right: Expected relative frequencies of nodal profiles, owing to differences in internode length. Bottom left: Calibres of the 53 internodes used in this study. Bottom right: Calibres of the corresponding 53 nodal profiles.

analysed separately for each animal; the results were congruent, and only pooled data are shown here.

The histogram of the sampled fibres showed a preponderance of thin and medium fibres when compared with a calibre histogram of 3661 fibres representative of the nerve's fibre population (Fig. 2). Nodal frequencies, however, do not parallel calibre frequencies because thicker fibres also have longer internodes. Expected nodal frequencies can be calculated from the nerve's calibre histogram, if each fibre class is reduced by a factor corresponding to its mean internode length. (The factors were obtained from a scatter diagram of internode length of 609 teased fibres from the same strain of mice.) The calibres of the 53 traced fibres correspond to the calculated maximum of nodal profiles (Fig. 2). Unfortunately we did not obtain any of the thicker fibres.

Dimensions of nodal and internodal profiles

The following parameters were routinely obtained: the area of the axon profile, the circumference of the axon profile, the diameter of a circle equivalent to measured area, the diameter of a circle equivalent to measured circumference (the preferred reference), and the non-circularity factor. The g-ratio of the 53 traced fibres was 0.70 ± 0.06.

Scatter diagrams of the non-circularity factors (Fig. 3) showed that nodal profiles were always more circular than internodal profiles; the majority (75%) of nodes had non-circularity factors between 0.8 and 0.95 (mean 0.86), consistent with Rydmark and Berthold (1983). The internodal segments of the same fibres were distinctly less circular (mean 0.73); 55% of them had factors of below 0.8. The scattergram of the 53 internodal profiles was roughly congruent with the scattergram of the 3661 fibres representing the nerve's population (mean 0.73). The latter had the skew to the left observed by Arbuthnott and co-workers (1980), Friede and Beuche (1985), and many others, as the thin fibres tended to be less circular than thick fibres. Different internodal non-circularities of thin or thick fibres had no recognizable effect on the non-circularities of their nodes.

The 53 fibres were arranged in order of increasing internodal diameters, and divided into six groups defined by 0.5 μm increments of circular axon diameter (Table 1). Our data confirm that the nodal segment of thick fibres is more deeply constricted than that of thin fibres as shown, among others, by the measurements of Rydmark (1981) or the longitudinal profiles of Raine (1982). For the six calibre classes in Table 1 the reduction in the area of the nodal profile

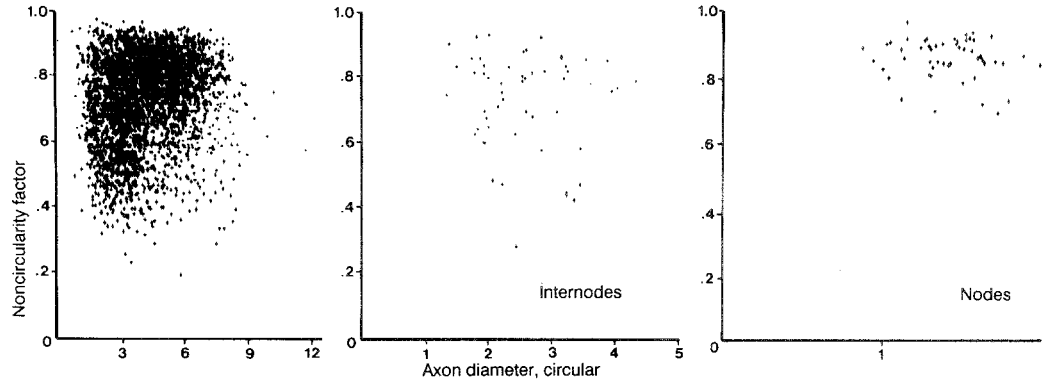


Fig. 3. Scatter diagram of the non-circularity factors in (left) 3661 fibres of the nerve, in the 53 internodes and in their corresponding nodes.

(node/internode) increased from 0.75, 0.46, 0.37, 0.32, 0.23 to 0.19. In terms of circular axon diameters, the degrees of constriction (node/internode) were 0.86, 0.68, 0.57, 0.56, 0.47 and 0.43. The values were practically identical with Fig. 6 of Rydmark (1981). As a consequence of the greater axonal narrowing in thick fibres, there was much less variation among the nodal areas than among the internodal areas: the former varied by a factor of 3, the latter by 15.

Nodal changes of neurofilaments and microtubules

The most obvious size-dependent variation among internodes was the well-known increase in the number of neurofilaments with increasing fibre calibre. Between the thinnest and thickest fibre class (Table 1) it amounted to a factor of 7.3. There was also an increase in internodal microtubules, although smaller, by a factor of 3.

The nodal constriction of the axon corresponded to a decrease in the number of neurofilaments but not of microtubules. Filament numbers decreased the more, the greater the axon's constriction. There was much variance in microtubule numbers, and most fibres had more microtubules at the node than in the internode. Aside from filaments and microtubules there was also a considerably increased density of vesicular and mitochondrial profiles (not measured) at the nodes. Smooth surfaced endoplasmic reticulum has been shown to be continuous across nodes (Tsukita & Ishikawa, 1976).

There were additional changes of nodal axoplasmic composition. An increase in nodal area was accompanied by increases in the number of both filaments and microtubules. However, unlike internodes, nodal profiles showed much greater increases in microtubules with calibre; in Table 1, the increase in microtubules between the thinnest and the thickest nodes (×3.6) was slightly greater than the increase in the number of filaments (×3).

In evaluating these changes one must consider the differences in fibre circularity between nodes and internodes, and their potential effect on the packing density of filaments or microtubules. Figure 4 shows the nodal and internodal axoplasmic packing densities of microtubules, of filaments and of their combined numbers for the six calibre classes. Densities are

Table 1. Axon area, neurofilaments and microtubules of corresponding internodal and nodal profiles

	Diameter of the internodal axon profile (μm)					
	<1.5 $n=3$	<2.0 $n=13$	<2.5 $n=12$	<3.0 $n=8$	<3.5 $n=12$	<4.24 $n=5$
Axon area, internode	1.39 ± 0.13	2.58 ± 0.26	4.0 ± 0.56	5.46 ± 0.47	8.05 ± 0.72	11.83 ± 1.41
Axon area, node	1.04 ± 0.21	1.19 ± 0.23	1.49 ± 0.49	1.72 ± 0.4	1.82 ± 0.48	2.2 ± 0.47
Neurofilaments, internode	259 ± 82	379 ± 135	639 ± 258	957 ± 187	1416 ± 418	1890 ± 732
Neurofilaments, node	167 ± 61	203 ± 102	285 ± 99	331 ± 79	456 ± 134	493 ± 222
Microtubuli, internode	38 ± 16	42 ± 16	50 ± 29	63 ± 19	95 ± 40	120 ± 22
Microtubuli, node	39 ± 14	58 ± 31	81 ± 38	98 ± 27	111 ± 55	139 ± 61

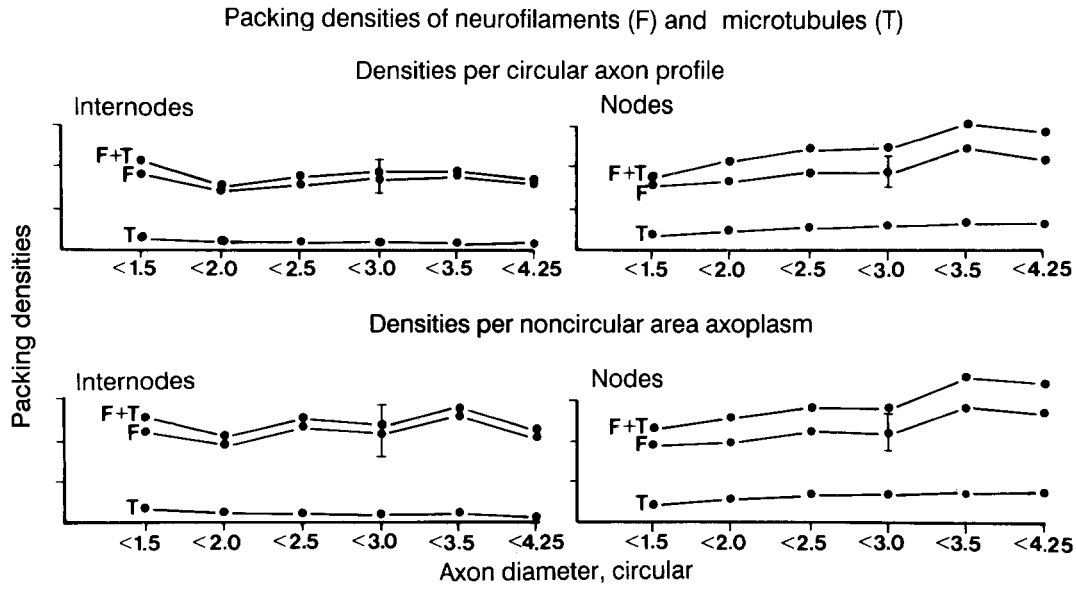


Fig. 4. Packing densities of neurofilaments and microtubules per area of axoplasm (10^2 per μm^2) for the six fibre classes shown in Table 1. Data are shown for the measured (non-circular) area of axoplasm, and corrected for non-circularity (based on fibre circumference). A single SD is shown to avoid clutter. Only the differences in tubular packing densities between nodes and internodes are statistically significant (see text).

shown for either the non-circular measured area of the axon profile or for the axon area corrected for circularity, eliminating the non-circularity factor. Both sets are similar. There were no statistically significant differrences in packing densities between internodes of different calibre. The nodes had significantly ($P < 0.005$ to < 0.001) greater densities of microtubules (increases approximately 500–700%) than the internodes. There was also a suggestion of moderate increase in nodal filament packing densities in the thicker fibres, consistent with the observations of Price and co-workers (1988a). However, even the maximum increase of 76% in the thick fibres had a P value of < 0.025 using a two-tailed t-test.

Some remarkable observations were made when analysing the microtubule data for the individual fibres: only four of the 53 fibres had equal numbers of tubules in the nodal and the internodal profile. Eight of the fibres had a deficit of eight to 87 tubules in the nodal profiles, averaging 26%. The majority of fibres ($n = 39$) had a mean 55% surplus of microtubules at the node. The remaining two fibres were not included in the 55% as they showed enormous increases in nodal microtubules: by 148% and 531% of the internodal numbers. We do not know if these two fibres were abnormal and, if so, in what way.

Another way to document the organization of the nodal cytoskeleton is in terms of the F/T (filament/microtubule) quotient. Except for three fibres with extreme deviations, the F/T quotients of the nodes ($n = 50$) were in a fairly narrow range of 3.71 ± 1.71. The six calibre classes showed no significant variation of the F/T quotient with nodal calibre. The F/T quotient of the internodes averaged 14.0 ± 11; its greater variation was caused by an increase of the quotient from 7.4 ± 3.0 for the thinnest to 16.0 ± 6.0 for the thickest fibre class.

A third way of analysing data is by scatter diagrams of the relation between the numbers of filaments or microtubules and circular axon area (Fig. 5). The scatter diagram for the internodes showed the familiar steep increase (slope: 175) in the number of filaments, accompanied by a smaller increase (slope: 9) in the number of microtubules. The nodes showed a still steeper increase (slope: 209) in filaments; the unique feature of nodes was a concurrent steep increase (slope: 53) and a great scatter of nodal microtubules. Correlation coefficients (r) were: internodal filaments: 0.87; nodal filaments: 0.66; internodal microtubules: 0.72; nodal microtubules: 0.55. Slopes similar to those in Fig. 5 are obtained (not shown) if one calculates filament and microtubule numbers per node and per internode from the areas and the densities tabulated for the six fibres by Berthold (1978).

Figure 4 also shows scatter diagrams of the sum of filaments plus microtubules *versus* circular axon area. These diagrams had higher correlation coefficients than the scatter diagrams for either filaments or for microtubules alone; the correlation coefficients (r) were 0.88 for the internodes and 0.72 for the nodes. This agrees with the calculations of Friede and Samorajski (1970) showing that F + T (the entire cytoskeleton) correlates best with axon calibre; this becomes particularly evident when the fractions of

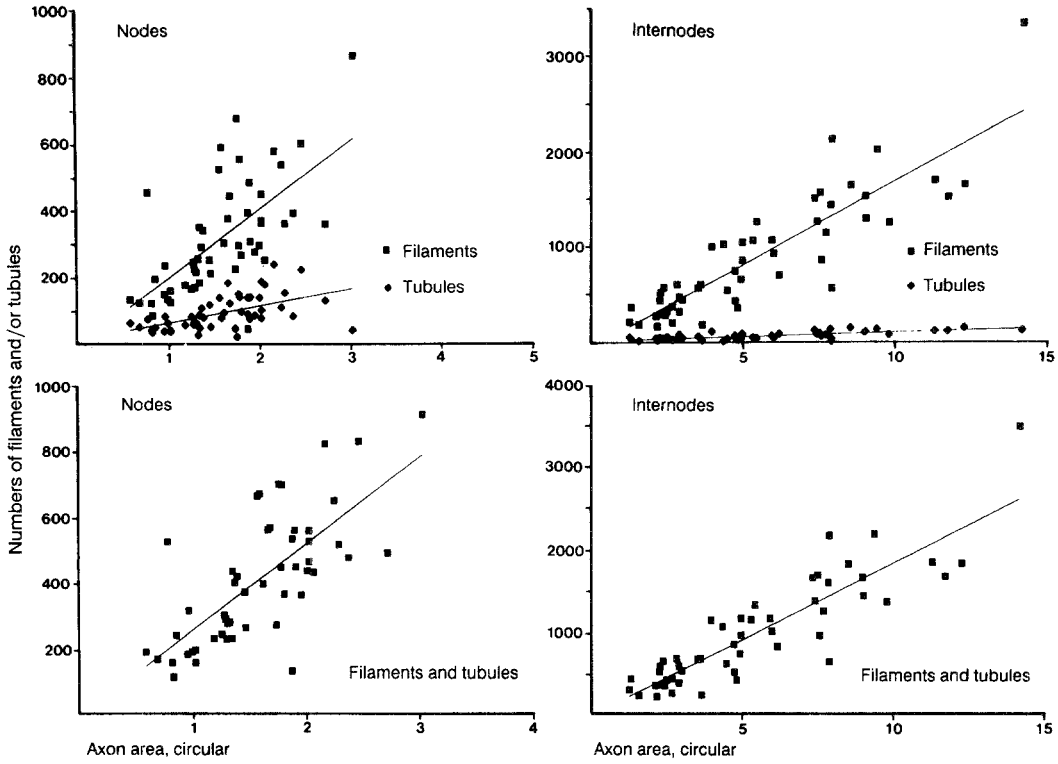


Fig. 5. Scatter diagrams of the numbers of filaments and tubules in nodes and internodes. The lower set shows the scatter diagram for the entire cytoskeleton (filaments plus microtubules). See text for correlation coefficients.

filaments and microtubules are subject to extreme variations.

We also plotted filaments, microtubules and their sum, against the non-circular axon area (not shown), i.e. without using the correction for non-circularity. This increased scatter, lowering the correlation coefficients. We considered this further indication that the axonal cytoskeleton should be assessed on the basis of circularity and not based on the packing densities observed in shrunken, non-circulár profiles.

Discussion

Our measurements confirm that the degree of axonal narrowing at the node is greater in thick fibres than in thin fibres (Rydmark, 1981; Raine, 1982). Our thickest fibre class narrowed to 19%, nearly identical with a reduction to less than 20% found by Rydmark (1981). The nodal narrowing of the axon corresponds to a striking decrease in the number of nodal neurofilaments (Tsukita & Ishikawa, 1981; Price *et al.*, 1988a), resulting in the well-known prevalence of microtubules in nodal profiles.

The internode-node restructuring of the axonal cytoskeleton, however, is not adequately described in terms of a reduction of filaments with roughly unchanged numbers of microtubules. Microtubule numbers varied independently and there were often more microtubules at the node than in the internode, as discussed later. Consequently, most fibres showed a marked decrease in the number of filaments and a slight increase in the number of microtubules at the node. The nodal cytoskeleton in fibres of different calibre, furthermore, showed increases of both filaments and in microtubules with calibre, but the rates of the increases were quite different from those observed in internodes. Consequently, there were highly significant increases in nodal packing density of microtubules, while the nodal packing density of filaments did not increase significantly.

Microtubules are widely recognized as important in the transport of axoplasmic organelles (Hirokawa & Yorifuji, 1986; Raine *et al.*, 1987). The concept of continuous tracks of microtubules from the perikaryon to the end of the fibre found initial support from data on equal numbers of microtubules in the stem fibre and in its branches (Weiss & Mayr, 1971; Nadelhaft, 1974; Letourneau, 1982) or stable numbers of tubules over a distance of 2 cm (Malbouisson *et al.*, 1985). However, serial sections disclosed a calculated average microtubule length of 370–760 μm (Tsukita & Ishikawa, 1981) or 108 μm (Bray & Bunge, 1981). Microtubules were seen to start or terminate in the axoplasm or at the plasma membrane, and also at axoplasmic organelles (Sasaki *et al.*, 1983). Moreover, the (+) end of axonal microtubules, the site of subunit

addition, is always distal to the cell (Heidemann *et al.*, 1981), so that growth is unlikely at the microtubule's proximal end near the perikaryon. Zenker and Hohberg (1973) calculated 11 times more microtubules in the terminal branches of a motor fibre than in its stem. Also, the stumps of transected fibres contained approximately ten times more microtubules distally than the stem of the fibre (Friede & Bischhausen, 1980).

We found that microtubules were clearly not continuous along the node-internode, and only four of the 53 fibres had equal counts for both regions. Eight fibres had a deficit of nodal microtubules, while 39 had a surplus averaging 55%. Investigator error was excluded by having different persons recheck the counts. It is also unlikely that pools of cold-soluble and cold-insoluble tubulin (Donoso, 1986; Sahenk & Brady, 1987) caused these differences, as nodal profiles showed either more or fewer tubules than the internode.

The nodal constriction of the axon's profile will affect axoplasmic transport. This is evident from nodal or paranodal changes in density of transported non-skeletal materials (Berthold & Mellström, 1982, 1986; Berthold *et al.*, 1986; Armstrong *et al.*, 1987; Zimmermann & Vogt, 1989). Transnodal transport of the components of the cytoskeleton, however, poses a more complex problem. There is ample evidence that the cytoskeleton polymers, particularly the neurofilaments, are instrumental in controlling the calibre of the axon (Friede & Samorajski, 1970; Friede, 1971; Lasek *et al.*, 1983; Hoffman *et al.*, 1985, 1987, 1988a,b). The restructuring of the nodal cytoskeleton, therefore, is not only relevant to the question of how transport is modified by the nodal constriction; it is even more critical to the understanding of how the nodal constriction is formed and maintained, and how cytoskeletal monomers and polymers interact locally to control the calibre of the fibre.

A slow proximodistal transport of a matrix of highly polymer cytoskeletal elements is well documented (Black & Lasek, 1980; Lasek *et al.*, 1984; Wujek *et al.*, 1986) with cotransport of the filaments and the microtubules within the same axoplasmic component (Hoffman & Lasek, 1980; Heriot *et al.*, 1985; Watson *et al.*, 1989). Nevertheless, certain inconsistencies in this concept were pointed out by Bamburg (1988). Transnodal cotransport of the components of the cytoskeleton needs to be reconciled with the observed changes in the nodal composition of the cytoskeleton. The data presented here allow four possibilities. First, a transnodal transport of freely moveable filaments (Price *et al.*, 1988b) would require that the filaments and the microtubules break all cross-linkage on passing the node (Hirokawa, 1982; Schnapp & Reese, 1982; Tsukita *et al.*, 1982; Hirokawa *et al.*, 1985; Meller, 1985). The filaments would need to navigate the node at multiples of their normal speed of transport, while microtubules would move normally or even slightly slower. A second possibility would involve stationary 'structural' and 'transport' cytoskeletal components (Nixon & Logvinenko, 1986; Matsumoto *et al.*, 1989), with only transport filaments passing the node. Third, there could be a selective depolymerization of filaments proximal to the node with distal repolymerization (Tsukita & Ishikawa, 1981). Finally, nodal and internodal axoplasm could have different equilibria between monomers and polymers of filaments (Morris & Lasek, 1984; Tashiro & Komiya, 1989; Ochs *et al.*, 1989), causing a shift of filament depolymerization within the nodal axon segment. The quantitative data provided here define the dimensions within which any of these four processes must operate to maintain nodal axon configuration.

Acknowledgements

The authors wish to thank Maria Thompson, Sabine Kübber, Robert Wagner and Stephan Löser for their excellent technical assistance. The study was supported by a grant from the Deutsche Forschungsgemeinschaft (Fr 609).

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