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# Greater number of microtubules per axon of unmyelinated fibers of mutant quails deficient in neurofilaments: possible compensation for the absence of neurofilaments

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**Abstract** Morphometric evaluations were performed on the peroneal nerve from mutant quails deficient in neurofilaments (NF) to elucidate the effect of an absence of NF on unmyelinated axons. The diameter frequency distribution of unmyelinated axons was similar between controls and mutants. The mean transverse axonal area, axonal circumference and circularity index of the unmyelinated axons were also similar in controls and mutants. However, the number of microtubules (MT) per axon was greater ($P<0.01$) in the mutants than in the controls. The regression analysis relating the number of MT per axon to the diameter of unmyelinated axons indicated a greater number of MT in the mutants than in the controls ($P<0.05$–$0.01$). A significantly greater number of MT per axon in the mutants may suggest a compensatory increase of MT in the absence of NF. This may conserve the size and transverse circular profile of the unmyelinated axons which are probably maintained by both MT and NF in the controls. The number of MT may be increased at the expense of the soluble fraction of tubulin.

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Neurofilament · Microtubule · Unmyelinated axon
Regression analysis

## Introduction

There is significant evidence to indicate that the number of neurofilaments (NF) in the axon is closely associated with the axon size [3, 4, 6, 7, 10, 11]. In mutant quails deficient in NF [13], there are no NF ultrastructurally in either myelinated or unmyelinated axons [19]. The diameters of a significant number of myelinated fibers of the proximal peroneal nerve only reach up to 7 μm in mutants, although they reach up to 10 μm in controls [20], indicating that the absence of NF may be critical for the determination of the axon size, especially of the large myelinated fibers which contain a large number of NF in controls. On the other hand, it is not known whether the unmyelinated axons, which in controls contain fewer NF than the myelinated axons, are altered morphometrically in the mutant quails deficient in NF. Therefore, this study was carried out to elucidate whether the axon size and circularity index of the unmyelinated fibers are altered in the mutants compared with the controls. Furthermore, a regression analysis between the number of microtubules (MT) or of MT plus NF (MT+NF) and the size of the unmyelinated axons was also performed to elucidate the effect of NF and MT on the size of the unmyelinated axons.

## Materials and methods

Mutant quails [9, 13, 19], three males and three females, and controls, three males and three females, of 8–12 weeks of age were perfused through the left cardiac ventricle with 3 % glutaraldehyde in 0.05 M cacodylate buffer (pH 7.4) at room temperature. Following perfusion fixation, part of the proximal peroneal nerve in the midthigh was removed, fixed in the same fixative for an additional 3 h, washed, osmicated in 1 % osmium tetroxide for

3 h, and processed for embedding into epoxy resin. Ultrathin transverse sections of each of the six mutant and six control nerves were cut. The electron micrographs of the unmyelinated fibers were taken systematically. On photographic enlargements (x 19,000), the lesser axonal diameter, the axonal circumference, the transverse axonal area and the circularity index (see Table 2) were obtained using programmed digitization and calculations [15]. All the MT and NF of each unmyelinated axon were counted under binoculars with low-power magnification [14]. Approximately 100 unmyelinated axons were analyzed for each nerve. Based on the data above, the number of MT, NF and MT+NF per axon and per $\mu m^2$ of the transverse axonal area (density) were calculated. A regression analysis between the relevant parameters was performed and the slope and intercept, and the intercepts for a given abscissa values were compared between controls and mutants [14]. Statistical evaluation was made with the Student's *t*-test.

## Results

### Qualitative findings

There was no evidence to suggest ongoing degeneration or loss of unmyelinated fibers in either controls or mutants. Qualitatively, the number of unmyelinated fibers seemed to be similar between controls and mutants. No NF were clearly identified in any of the unmyelinated axons in the mutants compared with the controls (Fig. 1).

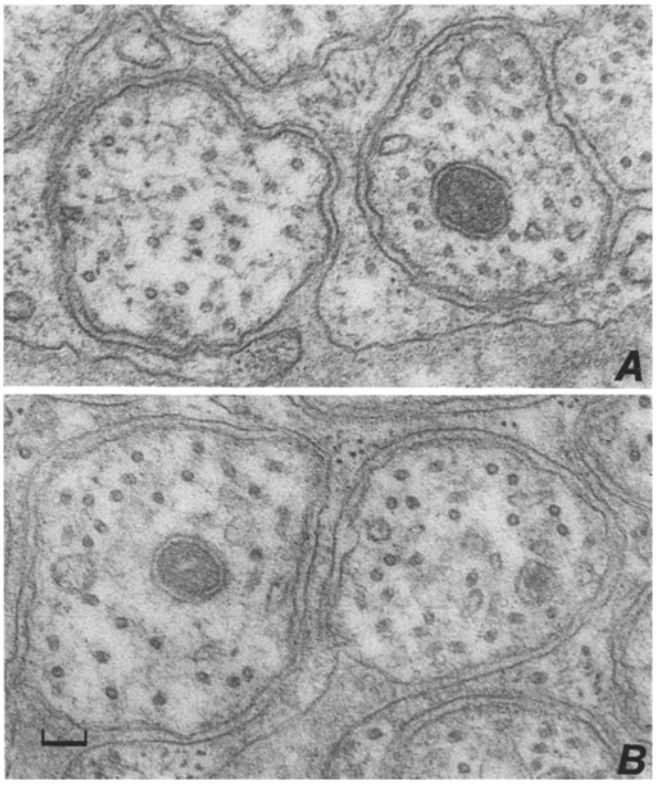


**Fig. 1** Electron micrographs of transverse sections of the unmyelinated axons from a control (**A**) and a mutant (**B**). Neurofilaments (NF) are clearly identified only in a control. *Bar* represents 0.1 μm

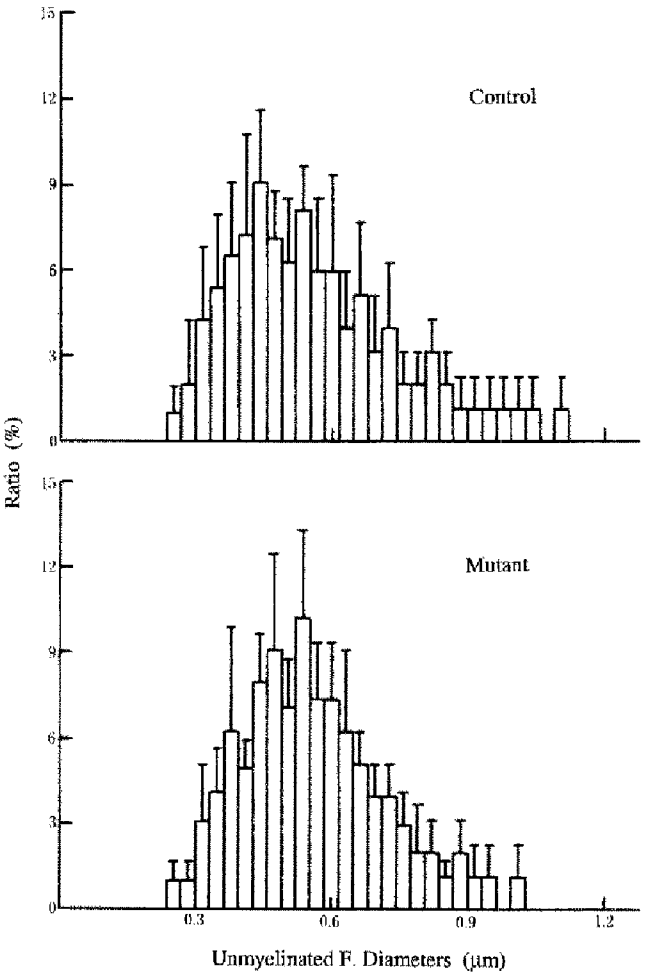


**Fig. 2** The composite histogram of the diameter frequency distributions of the unmyelinated fibers of the controls and mutants. *Bars* and *vertical lines* show means and their standard deviations, respectively. The distribution pattern is basically similar between the controls and the mutants

### Quantitative findings

#### *The size and circularity index of unmyelinated axons*

The composite histograms of the diameter frequency distributions of unmyelinated fibers of the controls and the mutants are shown in Fig. 2. The results seem to be basically similar in the controls and the mutants; however, the frequency of the larger diameter axons may be lower in the mutants. The median, mean, minimum and maximum diameters were almost identical for controls and mutants (Table 1). The transverse axonal area, axonal circumference and circularity index were also very similar in controls and mutants (Table 2).

#### *Numbers of MT, NF and MT+NF of unmyelinated axons*

The numbers of MT per axon were greater ($P<0.01$) in the mutants than in the controls, and those per $\mu m^2$ of

**Table 1** Diameter of unmyelinated fibers. Mean ± SD (*NS* not significant)

| | Diamaters (μm) | | | |
|---|---|---|---|---|
| | Median | Mean | Minimum | Maximum |
| Control ($n = 6$) | 0.54 ± 0.03 | 0.57 ± 0.03 | 0.25 ± 0.03 | 1.25 ± 0.13 |
| Mutant ($n = 6$) | 0.54 ± 0.02 | 0.57 ± 0.03 | 0.27 ± 0.04 | 1,15 ± 0.18 |
| Statistical significance | NS | NS | NS | NS |

**Table 2** Transverse axonal area, axonal circumference and circularity index of unmyelinated fibers. Mean ± SD (*NS* not significant)

| | Area (μm²) | Circumference (μm) | Circularity index[a] |
|---|---|---|---|
| Control ($n = 6$) | 0.46 ± 0.04 | 2.47 ± 0.13 | 0.94 ± 0.01 |
| Mutant ($n = 6$) | 0.46 ± 0.02 | 2.44 ± 0.66 | 0.96 ± 0.01 |
| Statistical significance | NS | NS | NS |

[a] $2\sqrt{\text{axonal area} \cdot \pi}$/axonal circumference

**Table 3** Numbers of microtubules (MT), neurofilaments (NF) and MT + NF in unmyelinated fibers. Mean ± SD (*NS* not significant)

| | Numbers per axon | | |
|---|---|---|---|
| | MT | NF | MT + NF |
| Control ($n = 6$) | 21 ± 2 | 5 ± 1 | 26 ± 3 |
| Mutant ($n = 6$) | 25 ± 1 | 0 | 25 ± 1 |
| Statistical significane | $p < 0.01$ | – | NS |
| | Numbers per μm² | | |
| | MT | NF | MT + NF |
| Control ($n = 6$) | 65 ± 6 | 16 ± 3 | 81 ± 9 |
| Mutant ($n = 6$) | 64 ± 3 | 0 | 64 ± 3 |
| Statistical significance | NS | – | $p < 0.01$ |

the transverse axonal area were similar between controls and mutants (Table 3). There were no NF in the mutants. The number of NF per axon and per μm² of the transverse axonal area were about 24 % of those of MT in the controls. The numbers of MT+NF per axon were not significantly different in controls and mutants (Table 3). On the other hand, the numbers of MT+NF per μm² of transverse axonal area were smaller ($P<0.01$) in the mutants than in the controls (Table 3).

### *Relationship between the number of MT per axon and the diameters or transverse axonal areas of unmyelinated fibers*

The linear relationships between the number of MT and the diameters or the transverse axonal areas of unmyelinated fibers were significant ($P<0.001$) in both the controls and mutants. In the regression analysis relating the number of MT to the diameters or the transverse axonal areas of unmyelinated fibers, the slope was significantly steeper for mutants than for controls ($P<0.05$), although there was no significant difference in the intercept between controls and mutants. The common lines relating the number of MT to the diameters for controls and mutants are shown in Fig. 3. The number of MT in mutants were greater ($P<0.05$–$0.01$) in the axons of any given diameter than that in controls (Fig. 3). Similar results were obtained for the common lines relating the number of MT to the transverse axonal areas. In the regression analysis relating the diameters or transverse axonal areas of unmyelinated fibers to the number of MT, both the slope and intercept were not statistically different between controls and mutants. The common lines relating the diameters to the number of MT for controls and mutants are shown in Fig. 4. The diameters were greater ($P<0.05$–$0.01$) in the controls than in the mutants for any given number of MT equal to or greater than 15. Similar results were obtained for the common lines relating the transverse axonal areas to the number of MT.

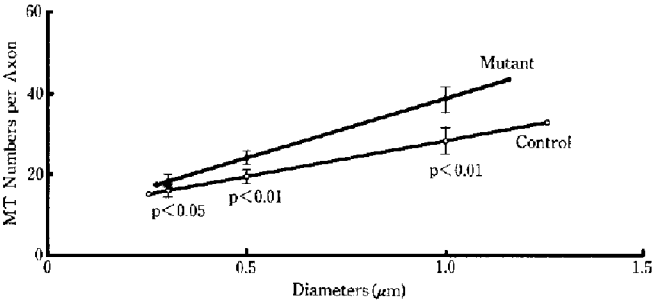


**Fig. 3** The common lines relating the number of microtubules (MT) per axon to the diameters of unmyelinated axons for the controls and mutants are shown. The ranges (mean ± SD) of MT numbers calculated from six regression lines for each of the controls and mutants are shown for the given diameter (0.3, 0.5 and 1.0 μm) with statistical evaluation. The number of MT is significantly greater in the mutants than in the controls for the any given diameter

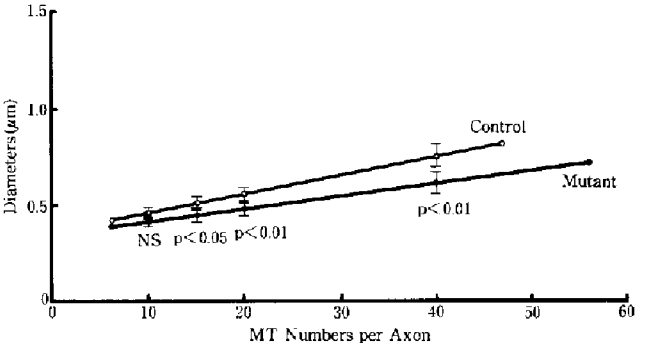


**Fig. 4** The common lines relating the diameters of unmyelinated axons to the MT numbers per axon for controls and mutants are shown. The ranges (mean ± SD) of the diameters calculated from six regression lines for each of the controls and mutants are shown for the given number of MT (10, 15, 20 and 40) with statistical evaluation. The diameters are significantly smaller in the mutants than in the controls for any number of equal to or greater than 15

*Relationship between the number of MT+NF per axon and the diameters or transverse axonal areas of unmyelinated fibers*

The linear relationships between the number of MT+NF and the diameters or the transverse axonal areas of unmyelinated fibers were significant ($P<0.001$) in both controls and mutants. In the regression analysis relating the number of MT+NF to the diameters or the transverse axonal areas of the unmyelinated fibers, both the slope and intercept were similar in controls and mutants. The common lines relating the number of MT+NF to the diameters for controls and mutants are shown in Fig. 5. There were no significant differences in the number of MT+NF at a given diameter between controls and mutants (Fig. 5). Similar results were obtained for the common lines relating the number of MT+NF to the transverse axonal areas. Similarly, in the regression analysis relating the diameters or the transverse axonal areas of unmyelinated fibers to the number of MT+NF, both the slope and intercept were similar between controls and mutants. The common lines relating the diameters for controls and mutants are shown in Fig. 6. There were no significant differences in the diameters at any given number of MT+NF between controls andmutants (Fig. 6). Similar results were obtained for the common lines relating the transverse axonal areas to the number of MT+NF.

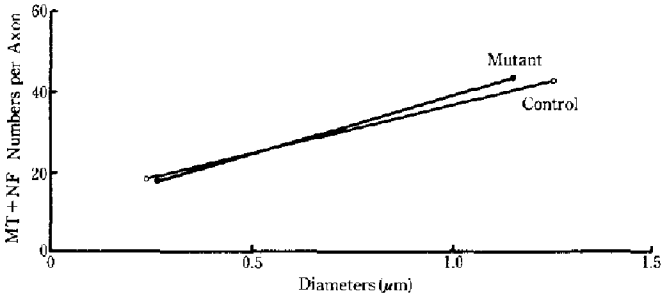


**Fig. 5** The common lines relating number of MT+NF per axon to the diameters of unmyelinated axons for controls and mutants are shown. The number of MT+NF calculated from six regression lines for each of the controls and mutants are similar for all given diameters (0.25 through 1.15 μm)

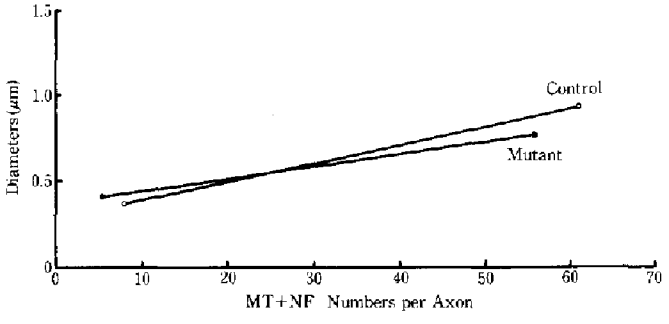


**Fig. 6** The common lines relating the diameters of unmyelinated axons to the MT+NF number per axon for controls and mutants are shown. The diameters calculated from six regression lines for each of the control and mutants are similar for all given number of MT+NF (8 through 56)

*Relationship between circularity index and transverse axonal areas*

In the regression analysis relating the circularity index to the transverse axonal areas, no significant correlation was found in either controls or mutants.

## Discussion

In the unmyelinated axons, MT and NF are the major axoplasmic organelles closely related to the axon size [3, 12, 16]. In the control quail, the mean number of NF per axon and per $\mu m^2$ of the transverse axonal area were about one fourth of those of MT (Table 3).

When the diameter, circumference and transverse area of the unmyelinated axons were compared between controls and mutants, all size parameters were similar. In addition, the diameter frequency distributions of unmyelinated fibers were basically similar in the controls and mutants. Therefore, it seems that the absence of NF does not drastically affect the size of the unmyelinated axons. In addition, the circularity index was both high and similar in controls and mutants, suggesting that the absence of NF has no significant effect on the maintenance of the transverse circular profile of the unmyelinated axons in the mutants compared with the controls.

On the other hand, the mean number of MT per axon was greater ($P<0.01$) in the mutants than in the controls. Furthermore, the regression analysis relating the number of MT to the diameters indicated a significantly greater number of MT in the axons, with all axon diameters between 0.27 and 1.15 μm, in the mutants than in the controls. Similarly, the regression analysis relating the axon diameters to the number of MT indicated that the diameters are significantly smaller in the mutants than in the controls when the number of MT is equal to or greater than 15. This suggests that the absence of NF may be responsible for the smaller diameter size of axons with 15 or more MT in mutants as compared with the controls, because in the controls the size reflects the presence of both MT and NF in the regression analysis [3, 12, 16]. However, no significant difference was found in the relationship between the diameters and the number of MT+NF between controls and mutants. Therefore, the significantly greater number of MT described above in the mutants may suggest an increase in MT to compensate for the absence of NF in the mutants, to conserve the size and transverse circular profile of unmyelinated axons [12, 16]. In the axons with MT numbers of 15 or greater, based on the results of the regression analysis, the compensatory increase of MT in mutants may not be enough to maintain the sizes to those of controls.

In this studies, the regression analysis of the relationship between MT or MT+NF numbers and axon diameters in mutants and controls proved to be more useful

than the evaluation of the mean value of each parameter for detecting the altered relationship.

As suggested in the introduction, the effect on an absence of NF on the unmyelinated axons seems to be different from that on the myelinated axons based on the light microscopic data [20]. However, the study of such effects on the myelinated axons using electron microscopy provides a more systematic quantitative analysis compared with that on the unmyelinated axons, because the myelinated axons show a greater variability of the axon size and the numbers of MT and NF than the unmyelinated axons. Therefore, such a study on the myelinated axons is in progress.

What is the mechanism which causes the increase of MT in the unmyelinated axons? MTs form one of the major cytoskeletons involved in the growth and maintenance of neurites and axoplasmic transport [2, 12, 16], and there is a significant correlation between the axon diameters and the numbers of MT or NF, or both, in unmyelinated and myelinated axons in the limb nerves of rats and mice [3]. On the other hand, the microtubular protein tubulin is composed of two, detergent-soluble (unpolymerized) and detergent-insoluble (polymerized), fractions. In dissociated and explant cultures of rat sympathetic neurons, following detergent extraction under MT-stabilizing conditions, 30 % of the tubulin was unpolymerized [1]. Experimentally, the number of MT of unmyelinated axons after the reassembly of cold depolymerized MT exceeds the initial number of MT [5]. Moreover, it is known that taxol application increases the number of MT in axons and cytoplasms of Schwann cells and endothelial cells in the peripheral nerve of rats [17, 18] and that zinc treatment results in a remarkable structural preservation of MT after fixation with osmium [8]. Therefore, considering the dynamic alteration of the two fractions of microtubular protein in the various conditions above, we speculate that in the mutants, the absence of NF may be compensated by the increase of the number of MT at the expense of soluble fractions of tubulin possibly in the form of unpolymerized dimer and oligomer [2] in the unmyelinated axons to maintain their structural integrity. In addition, the soluble fractions of tubulin may be decreased in the mutants to increase the polymerized MT. However, we cannot exclude the possibility that the total amount of tubulin may be increased to compensate the absence of NF, retaining the ratio of the two fractions or altering the equilibrium condition of the two fractions [1, 2].

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