

# Control of Axonal Caliber by Neurofilament Transport

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**ABSTRACT** The role of neurofilaments, the intermediate filaments of nerve cells, has been conjectural. Previous morphological studies have suggested a close relationship between neurofilament content and axonal caliber. In this study, the regenerating neuron was used as a model system for testing the hypotheses that neurofilaments are intrinsic determinants of axonal caliber, and that neurofilament content is controlled by the axonal transport of neurofilaments. This system was chosen because previous studies had shown that, after axotomy, axonal caliber was reduced within the proximal stump of the regenerating nerve and, because the relative amount of neurofilament protein undergoing axonal transport in regenerating axons was selectively reduced.

The relationship between axonal caliber and neurofilament number was examined in a systematic fashion in both regenerating and control motor axons in rat L5 ventral root. Reconstruction of the spatial and temporal sequences of axonal atrophy in the proximal stump after axotomy showed that reductions in axonal caliber were first detected in the most proximal region of the root and subsequently progressed in a proximal-to-distal direction at a rate of 1.7 mm/day, which is identical to the rate of neurofilament transport in these neurons. Quantitative ultrastructural studies showed that these reductions in caliber correlated with a proportional decrease in the number of axonal neurofilaments but not microtubules.

These results support the hypotheses that neurofilament content is a major intrinsic determinant of axonal caliber and that neurofilament content is controlled by the axonal transport of neurofilaments. On this basis, we suggest a role for neurofilaments in the control of axonal volume.

Intermediate filaments are prominent components of the cytoskeleton in a variety of cell types. In some settings, functional roles are suggested by their localization, e.g., the “anchoring” of intermediate filaments in the dense plaque of smooth muscle cells (1). These filaments are thought to influence cell size, shape, and structural stability and to organize intracellular space. Two specific roles have been proposed for neurofilaments (the intermediate filaments of nerve cells). One is in intracellular motility or axonal transport. Roles in the mechanisms of both slow (2) and fast (3) axonal transport now appear unlikely. In particular, recent studies have shown that fast transport can take place in microtubule “channels” essentially devoid of neurofilaments (4, 5). Another suggested role for neurofilaments is to influence the degree of stability (as opposed to plasticity) of neurons and their processes (6, 7). This hypothesis was suggested by the paucity of neurofilaments in highly “plastic” developing and regenerating neurite

processes and the increase in neurofilament content seen with maturation (8).

In this report, we suggest that one role of neurofilaments is to determine axonal caliber, and that neurofilament content is controlled through the delivery of neurofilaments by axonal transport. These hypotheses were suggested by a variety of previous morphological studies which demonstrated a close correlation between neurofilament number and axonal caliber (9–11), by the observation that neurofilament proteins are synthesized exclusively in the cell body and delivered to the axon by slow axonal transport (2), by studies suggesting that almost all of the neurofilament protein in the axon is present in polymeric (filamentous) form (7), by the observation that turnover of neurofilaments within the axon is small (12), and by experimental models in which blockade of neurofilament transport results in changes in axonal caliber (13).

In most experimental systems, a critical test of the role of

neurofilament transport in the control of axonal caliber is hampered by parallel changes in content or delivery of other cytoskeletal proteins, membranous organelles, or other axonal constituents. This is not a problem in the case of regenerating neurons. The amount of neurofilament protein transported in the proximal stumps of regenerating axons is selectively reduced compared to other cytoskeletal proteins (14), and there is relatively little alteration in the rapid axonal transport of membranous organelles and other axonal constituents in regenerating neurons (15–17). In this study, the spatial and temporal sequences of changes in caliber and neurofilament content were examined in the proximal stumps of transected rat sciatic motor fibers. We related these changes to previously observed alterations in the axonal transport of neurofilament proteins in these neurons.

After transection of a peripheral axon, the portion disconnected from the cell body (the distal stump) degenerates. Regeneration of axons and reinnervation of targets in the periphery occur as a result of outgrowth (elongation) of axonal sprouts from the cut end of the proximal stump (18). After axonal transection, the caliber of individual axons in the proximal stump is reduced, as shown by classical and recent morphological and electrophysiological studies (19–25). Restoration of caliber has been correlated with successful reconnection of regenerating axons with targets in the periphery (26).

The decrease in the amount of labeled neurofilament protein transported in the proximal stump of regenerating motor fibers (14) suggested the possibility that the decline in axonal caliber proximal to the site of nerve transection might correlate with the reduction in neurofilament transport. If this occurred, it should evolve in a proximal-to-distal sequence at a rate comparable to the velocity of neurofilament transport (1–2 mm/d). In addition, these changes in axonal caliber should be accompanied by reductions in the number of neurofilaments within these axons without major alterations in the proportion of axonal area occupied by other axoplasmic constituents. In the present study, we used morphometric methods to examine systematically the spatial and temporal evolution of the reduction in caliber of motor axons within the L5 ventral root after crushing the sciatic nerve. Changes in the caliber of individual axons were correlated with the number of neurofilaments within these axons.

## MATERIALS AND METHODS

**Surgical Procedures:** Surgical procedures were carried out using chloral hydrate anesthesia (400 mg/kg, i.p.). The sciatic nerves of 7-wk-old male Sprague-Dawley rats were either crushed or cut and ligated at the junction of the L4 and L5 ventral roots (at a distance of ~50 mm from the spinal cord). We examined the caliber of motor axons in the L5 ventral root between 1 and 10 wk after injury, i.e., in animals 8–17 wk of age. We also examined the caliber in unoperated control animals 8, 12, and 18 wks of age. Nerves were crushed twice for 30 s using fine watchmaker's forceps. In the cases where the sciatic nerve was cut, it was ligated with a 6-0 black silk suture immediately proximal to the site of transection (to minimize outgrowth and reinnervation), and a 10-mm segment of the nerve was resected distally. Equal numbers of right and left sciatic nerves were lesioned. Only one nerve was operated in each animal. We obtained control nerves from unoperated, age-matched animals.

**Tissue Preparation:** Animals were anesthetized using chloral hydrate and perfused through the ascending aorta with 0.9% saline followed by 5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3) at room temperature. After the perfused animals were stored overnight (18 h) at 4°C, L5 ventral roots were removed and placed in 0.1 M phosphate buffer (pH 7.3). Segments (3 mm) were obtained from the proximal, middle, and distal regions of these roots, which extended 0–3, 12–15, and 24–27 mm from the spinal cord, respectively.

After postfixation in 2% osmium tetroxide, these segments were dehydrated in alcohol and embedded in Epon. Transverse sections (1- $\mu$ m thick), stained with toluidine blue, were examined using light microscopy. We examined thin sections (pale silver interference color), stained with uranyl acetate and lead citrate, with a Hitachi model H-600 electron microscope (Hitachi America, Ltd., Allendale, NJ).

**Measurement of Axonal Caliber:** Axonal caliber was measured at the proximal, middle, and distal levels of the roots at weekly intervals after nerve injury. Several roots (from several different animals) were analyzed at each time interval. Four nonoverlapping fields in each section were photographed at a magnification of  $\times 250$  using a Zeiss Photomicroscope II (Carl Zeiss, Inc., New York) and printed at a final magnification of  $\times 2,000$ . Axonal areas and circumferences were measured from these prints using a Hewlett-Packard model 9864 digitizer interfaced with a model 9815s programmable calculator (Hewlett-Packard Co., Palo Alto, CA). All myelinated axons in these fields (except those sectioned through nodes of Ranvier, paranodal regions, and Schmidt-Lantermann clefts) were measured. Axonal circularity was calculated using the parameter  $\phi$ , which is defined as the ratio of axonal area to the area of a circle having the same circumference as the axon (27). Axonal diameters were calculated from measured areas. The percentage of axons in each size (diameter) class was determined at three levels of each root. These values were used to calculate the mean percentage of axons in each size class at a given level for all of the roots examined at a particular time interval. Histograms were constructed by plotting these mean percentages as a function of axonal diameter. Differences between these histograms, e.g., those for the same level at different times after injury, were evaluated using the  $\chi^2$  test to compare the proportion of axons in one histogram with diameters greater than or equal to the median diameter in the other. By definition, 50% of the axons in the latter had diameters greater than or equal to the median. This analysis ( $\chi^2$  test) was not carried out if there was significant variability among the data from the individual roots within each set; variability was assessed using the Mantel-Haenszel test. In the course of this study, slightly  $>65,000$  axons from  $>40$  animals were analyzed.

### *Quantitation of Axons in Regenerating and Control*

**Roots:** The total number of myelinated axons in regenerating and contralateral control roots was counted at all three levels between 1 and 3 wk after crush. Photographs of these roots were printed at a final magnification of  $\times 400$ .

### *Quantitation of Axonal Microtubules and Neurofilaments:*

These studies were carried out using a single animal killed 3 wk after one sciatic nerve was crushed. Thin sections from the proximal levels of the regenerating and contralateral control roots were placed on Formvar-coated grids. Through the use of electron microscopy, all of the axons in both roots were photographed in overlapping fields at a magnification of  $\times 2,000$ . Axons obscured by grid bars were photographed in the adjacent serial section. These photographs were printed at a final magnification of  $\times 5,000$ , and the areas of the myelinated axons were measured. In this way, axons in the regenerating root could be selected for analysis along with axons of comparable area in the control root (for selection criteria, see Results). These axons were reidentified, photographed ( $\times 30,000$ ), and reconstructed using montages (final magnification,  $\times 75,000$ ). An additional higher-magnification photograph, usually  $\times 90,000$ , was taken of a region of the myelin sheath in which the lamellae could be well visualized for counting.

Using these montages, the total areas of these axons, as well as the areas occupied by membranous structures (i.e., mitochondria, vesicles, and agranular reticulum), were measured using the digitizer. Because we found that microtubules and neurofilaments were not uniformly distributed throughout the axoplasm, it was necessary to count all the microtubules and neurofilaments in each axon. In the majority of cases, these structures were sectioned transversely, making them easy to identify. Microtubules and neurofilaments sectioned obliquely were only counted if they could be unequivocally distinguished from other constituents. The frequency of these obliquely sectioned microtubules and neurofilaments was equally low in regenerating and control axons. All counts were done by either of two observers. Tests of reproducibility revealed that counts of the same region of axoplasm by a single observer on two occasions or by each of the separate observers varied by  $<5\%$ . A total of 89,160 neurofilaments and 16,640 microtubules were counted in 22 regenerating and 15 control axons. The largest axon (area = 59  $\mu\text{m}^2$ ) contained 6,934 neurofilaments and 817 microtubules.

## RESULTS

### *Motor Axons in Control Ventral Roots*

Motor axons in the L5 ventral root normally fall into two distinct size classes (Fig. 1*a*). Large axons, which include the

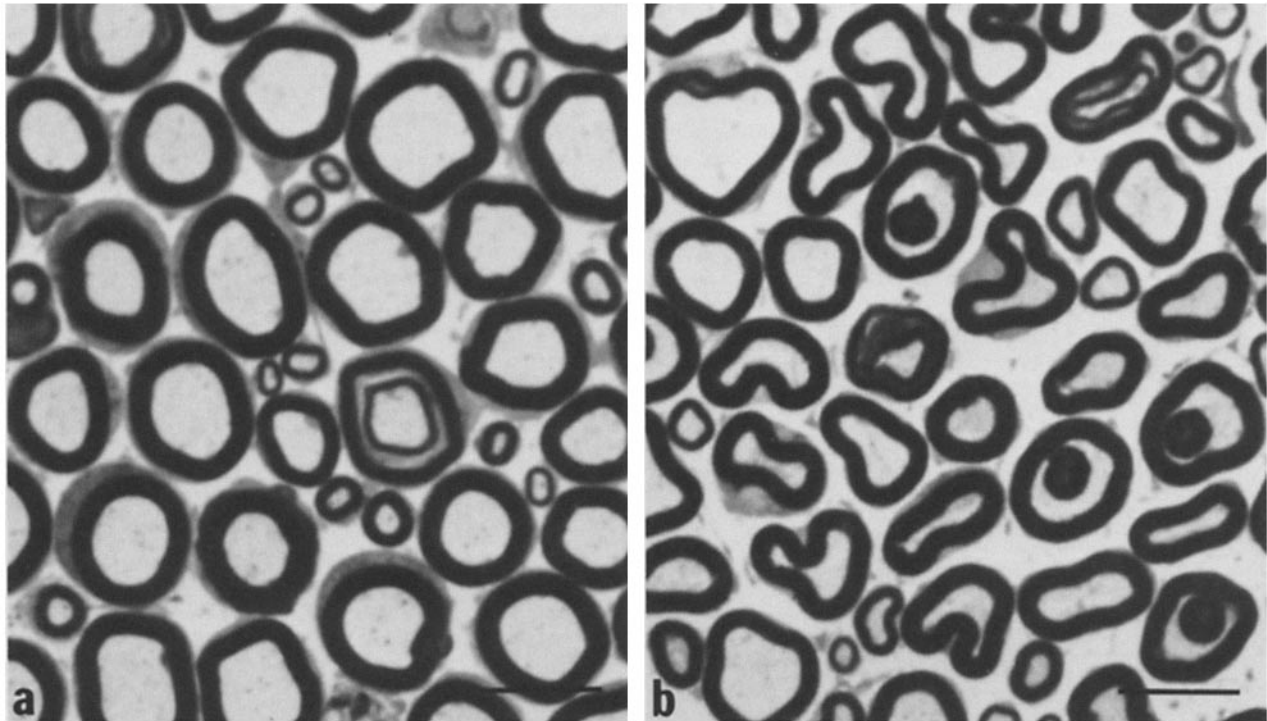


FIGURE 1 Changes in axonal size and shape during regeneration. These light micrographs compare cross sections of axons at proximal levels of control (a) and regenerating (b) L5 ventral roots 4 wk after crushing the sciatic nerve. Axons are the pale-staining disks surrounded by darkly stained rings (myelin sheaths). The control root was obtained from an unoperated 12-wk-old animal. Note the bimodal distribution of fiber sizes in both a and b. Note the decrease in caliber and loss of circularity in the regenerating fibers. Epon sections, toluidine blue. Bars, 10  $\mu\text{m}$ .  $\times 1,600$ .

alpha efferents, account for approximately two-thirds of the myelinated fibers in the root (Figs. 1 a and 2). Small axons, i.e., the remaining one-third, largely correspond to the gamma efferents. Between 8 and 18 wk of age, large axons undergo significant growth in caliber, while small axons grow much less (Fig. 2). During this growth, caliber is indistinguishable at the proximal and distal levels of the root (i.e., over a distance of  $\sim 25$  mm) (Fig. 2). Normally, these axons are relatively round (Fig. 1 a), and there is a well-defined relationship between caliber and myelin sheath thickness. As we shall demonstrate later, reductions in caliber during regeneration were associated with a loss of circularity and the presence of inappropriately thick myelin sheaths (Fig. 1 b); in regenerating roots, this allowed identification of axons that had undergone reductions in caliber.

#### Progression of Changes in the Caliber of Regenerating Motor Axons

The caliber of motor axons in the L5 ventral root is significantly reduced between 2 and 5 wk after crushing the sciatic nerve (Fig. 3, and see Fig. 5). Using the  $\chi^2$  test to compare the histograms from corresponding levels of regenerating and control roots, the first statistically significant reduction in caliber was found 2 wk after crush at the proximal level ( $P < 0.001$ ) (Fig. 3). At three weeks, axonal caliber in regenerating roots was significantly different from controls at both the proximal and middle levels ( $P < 0.001$ ) but not distally (Fig. 4). 4 wk after crush, significant differences were also found distally ( $P < 0.001$ ) (Fig. 3). These differences were characterized by a marked decrease in the caliber of large axons, which resulted in loss of the relatively well-defined

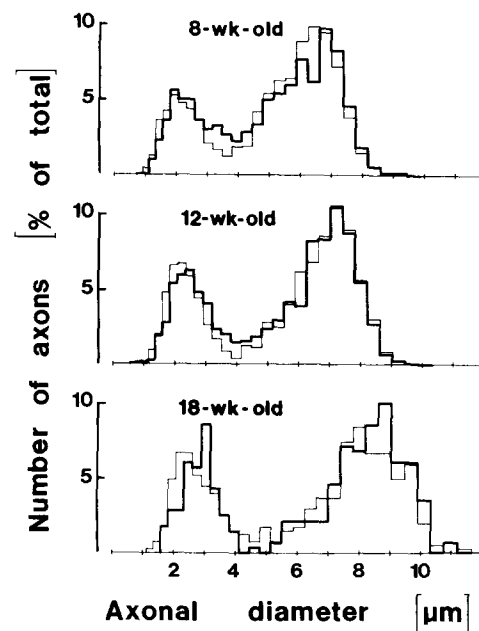


FIGURE 2 Growth of control axons. These histograms compare axonal caliber at the proximal (bold lines) and distal (thin lines) levels of the L5 ventral root in unoperated 8-, 12-, and 18-wk-old animals. The mean number of axons in each size class, expressed as a percentage of the total, is plotted as a function of axonal diameter. Three roots were analyzed at 8 and 12 wk and one root at 18 wk.  $\sim 400$  axons were analyzed in each root.

separation between the two size classes. This was most apparent at 4 and 5 wk after crush, when the caliber of large axons was maximally reduced (Figs. 3 and 5, respectively).

This apparent shift in the caliber of large axons to smaller sizes did not result from selective loss of large axons, in that there was no morphological evidence of axonal degeneration in ventral roots. To confirm that there was no axonal loss in regenerating roots, we compared the total number of myeli-

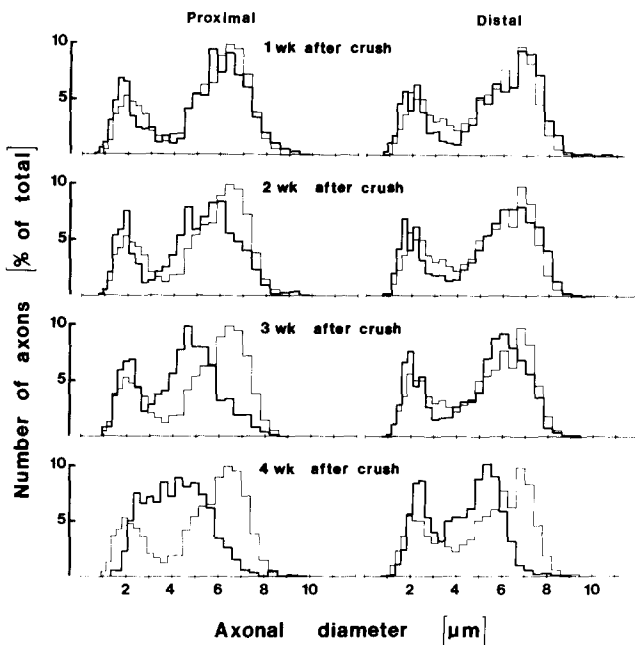


FIGURE 3 Reductions in caliber during regeneration. Axonal caliber is compared in regenerating (bold lines) and control (thin lines) axons at the proximal and distal levels of the L5 ventral root 1, 2, 3, and 4 wk after sciatic nerve crush. Control roots were obtained from unoperated 8-wk-old animals. Three roots were analyzed in each case. ~400 axons were analyzed in each root.

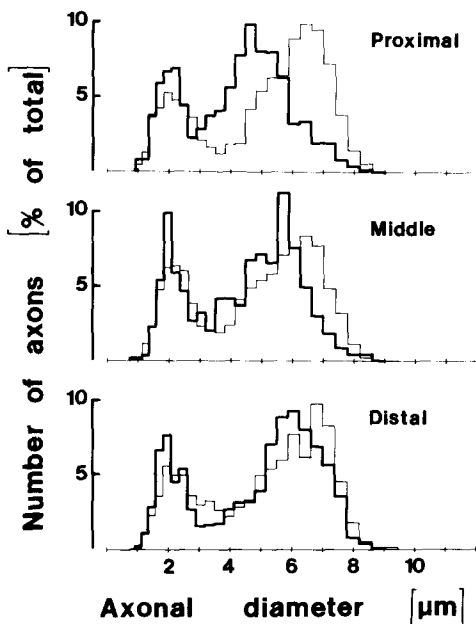


FIGURE 4 Spatial evolution of changes in caliber along regenerating axons. Caliber is compared in regenerating (bold lines) and control (thin lines) axons at the proximal, middle, and distal levels of the L5 ventral root 3 wk after sciatic nerve crush. Control roots were obtained from unoperated 8-wk-old animals. Three roots were analyzed in each case. ~400 axons were analyzed in each root.

nated axons in the L5 ventral roots of both regenerating and unoperated contralateral nerves at 1, 2, and 3 wk after crush. These analyses, carried out at all three levels of the root in three animals at each postcrush interval, revealed no change in the number of myelinated axons in the root after nerve crush injury. For example, 3 wk after crush, the mean number of myelinated fibers in the roots was  $1,656 \pm 37$  ( $n = 8$ ) for regenerating nerves as compared with  $1,646 \pm 20$  ( $n = 9$ ) for contralateral control nerves (Table 1).

The  $\chi^2$  test revealed statistically significant reductions in the caliber of regenerating axons at the proximal level of roots 2 wk after crush, at the middle level at 3 wk, and distally at 4 wk. Because the distance between these levels was known (i.e., 12 mm), it was possible to calculate that this reduction in the caliber of larger axons proceeded in a proximal-to-distal direction along the roots at a rate of 1.7 mm/d, a rate identical to the velocity of neurofilament transport in these axons (2).

### Restoration of Caliber in Regenerating Axons

After crush injury, restoration of caliber was correlated temporally with the recovery of motor function. Presumably, this functional recovery reflected effective regeneration and reinnervation of muscle fibers in the leg and foot. The first significant increases in caliber were found 8 wk after crush at both the proximal and distal levels (Fig. 5). The apparent increase in caliber at the proximal level 6 wk after crush was not statistically significant (Fig. 5). Thus, the data did not allow us to determine whether caliber was restored proximally before it was distally. Presumably, this reflects the nonsynchronous reinnervation of muscle fibers located at different distances from the crush site with restoration of caliber occurring first in axons reinnervating proximal muscles.

In nerves that were cut and ligated (rather than crushed), restoration of caliber was not observed at either 6 or 8 wk after axotomy (Fig. 6). Recovery of motor function was also absent in these animals. Thus, in axons where regeneration and/or reconnection was prevented, caliber was not restored.

### Quantitation of Changes in the Caliber of Regenerating Axons

The magnitude of changes in the caliber of large motor axons was estimated by measuring the mean area of the largest two-thirds of axons in regenerating and age-matched control roots (Fig. 7). This analysis revealed that the mean area of control axons, which, as we have already seen, was equal at the proximal and distal levels of the roots, increased at a rate of  $1.5 \mu\text{m}^2/\text{wk}$  between 8 and 18 wk of age. In regenerating axons, reductions in area at the proximal level preceded those

TABLE 1  
Numbers of Axons in Regenerating and Contralateral Control Roots

Postcrush time interval	Number of axons*	
	Regenerate	Control
wk		
1	$1,562 \pm 87$ (7)	$1,593 \pm 73$ (9)
2	$1,303 \pm 55$ (9)	$1,372 \pm 38$ (9)
3	$1,656 \pm 37$ (8)	$1,646 \pm 20$ (9)
Overall	$1,507 \pm 24$ (24)	$1,537 \pm 27$ (27)

\* All values shown as means  $\pm$  SEM; number of observations in parentheses.

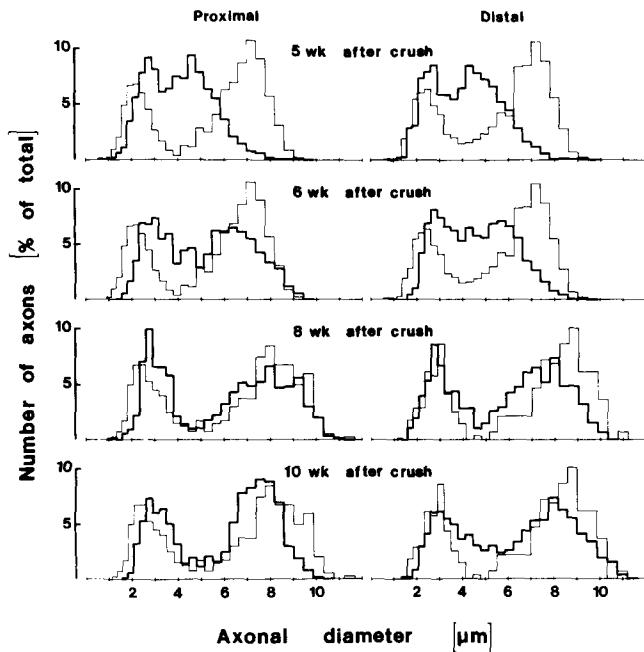


FIGURE 5 Recovery of caliber following nerve crush. Caliber is compared in regenerating (bold lines) and control (thin lines) axons at the proximal and distal levels of the L5 ventral root 5, 6, 8, and 10 wk after sciatic nerve crush. Control roots were obtained from unoperated 12-wk-old animals (for comparison with 5- and 6-wk regenerates) and from 18-wk-old animals (for comparison with 8- and 10-wk regenerates). Three roots were analyzed in the 12-wk-old control animals, as well as at 5, 6, and 10 wk after crush. Two roots were analyzed at 8 wk after crush, and one root was analyzed in 18-wk-old controls. ~400 axons were analyzed in each root.

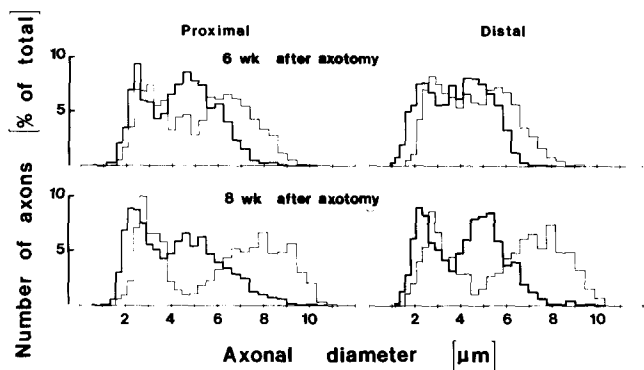


FIGURE 6 Recovery of caliber after sciatic nerve crush vs. cut. The caliber of regenerating axons is compared in the L5 ventral root (proximal and distal levels), which were either cut and ligated (bold lines) or simply crushed (thin lines). In both cases, nerves were examined at 6 and 8 wk after injury. Three roots were examined from cut and ligated nerves at 6 and 8 wk, as well as from crushed nerves at 6 wk. Two roots were examined from crushed nerves at 8 wk. ~400 axons were analyzed in each root.

distally. Yet, the final magnitudes and rates of these reductions were comparable at both levels. In both cases, the initial rate of decline ( $5\text{--}6 \mu\text{m}^2/\text{wk}$ ), i.e., between 1 and 3 wk at the proximal level and between 2 and 4 wk distally, was fivefold greater than the final rate ( $1 \mu\text{m}^2/\text{wk}$ ), i.e., between 3 and 5 wk proximally and between 4 and 5 wk distally. Restoration of caliber after crush injury occurred at a similar rate at both levels between 5 and 8 wk ( $7 \mu\text{m}^2/\text{wk}$ ). At 8 and 10 wk after

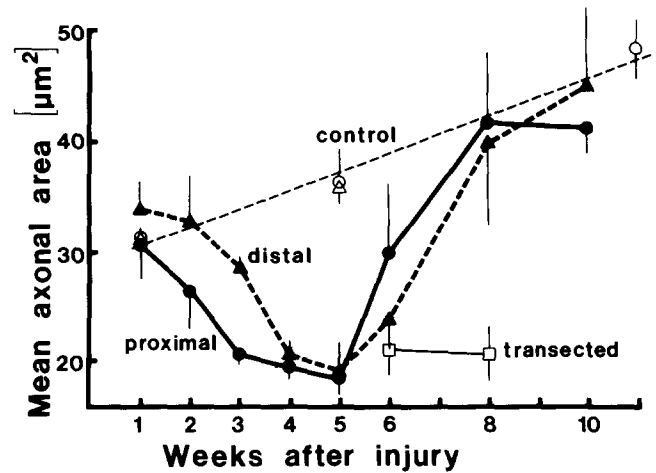


FIGURE 7 Time course of changes in the caliber of regenerating axons. This graph illustrates changes in the mean area of large axons in the L5 ventral root during normal growth, after crushing the sciatic nerve, and after cutting and ligating the nerve. The mean areas of the two-thirds largest axons at the proximal and distal levels are plotted as a function of time after injury. Normal growth at the proximal (○) and distal (△) levels of roots is illustrated in unoperated animals 8, 12, and 18 wk of age. Changes in axonal area are compared at the proximal (●) and distal (▲) levels of the root after nerve crush, as well as at the proximal level 6 and 8 wk after cutting and ligating the nerve (□). Each data point represents the mean of three values except in crushed nerves at 8 wk and control nerves at 11 wk (18-wk-old controls) where only two roots were examined. Standard errors are indicated by vertical bars.

crush, mean axonal areas in the regenerating roots were comparable to those anticipated for controls.

Restoration of caliber was not observed after the nerve was cut and ligated (Fig. 7). At 6 and 8 wk after nerve cut, mean axonal areas were similar to those found in crushed nerves when they were maximally reduced, i.e., at 5 wk. By 8 wk, mean axonal area was markedly different in cut nerves (where it remained maximally reduced) and crushed nerves (where significant restoration of caliber had occurred) (Fig. 7). Thus, restoration of caliber was not observed in axons which were prevented from reconnection with the periphery by ligating the nerve after it was cut.

#### Loss of Axonal Circularity as an Index of Reductions in Caliber

We found that axons which had undergone reductions in caliber became less circular in shape (Fig. 1). These changes were quantitated in a regenerating and control root 4 wk after crush using the parameter  $\phi$ , which is defined as the ratio of axonal area to the area of the circle having the same circumference as the axon (27).  $\phi$  has a maximum value of 1.0 for circular axons and declines as circularity is reduced. In the control root, axons were clustered into two major size classes (small and large) with mean areas of  $3.5$  and  $32.9 \mu\text{m}^2$  and  $\phi$  values of 0.78 and 0.90, respectively (Fig. 8). In contrast, the regenerating root contained a large number of axons with lower  $\phi$  values and areas intermediate between those of the two size classes in the control root (Fig. 8). It seemed likely that this new population, unique to the regenerating root, represented large axons which have undergone reductions in area.

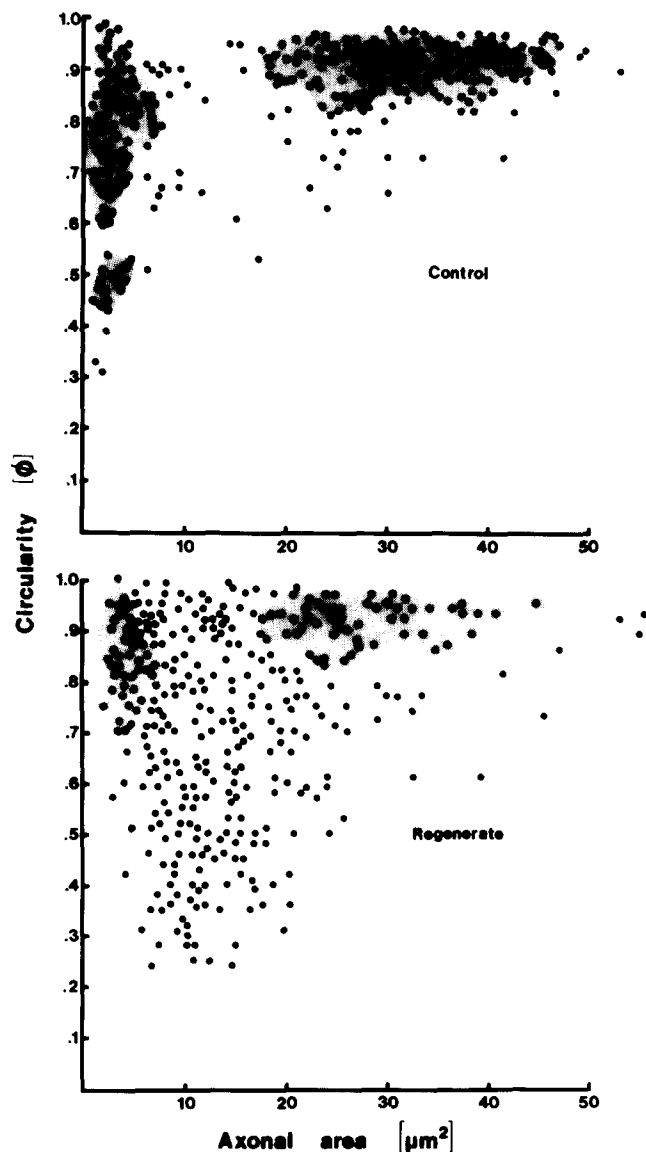


FIGURE 8 Changes in the circularity of regenerating axons. The circularity of axons at the proximal level of regenerating (lower profile) and control (upper profile) L5 ventral roots are compared 4 wk after crushing the sciatic nerve. The control root was analyzed in an unoperated 12-wk-old animal. Axonal circularity was measured using the parameter  $\phi$  (ratio of axonal area to the area of a circle having the same circumference as the axon). When  $\phi$  is plotted as a function of axonal area, axons in the control root are clustered into two size classes (stippled areas, upper profile). To facilitate comparison, the same regions are also stippled in the lower profile.

To investigate this possibility further, we compared myelin sheath thickness in regenerating and control axons with similar areas but different  $\phi$  values (Fig. 9). Previous studies in normal fibers have shown that myelin sheath thickness (expressed as the number of myelin lamellae) is directly correlated with axonal area for axons  $<20 \mu\text{m}^2$ . When axonal area was reduced, the number of myelin lamellae remained relatively unchanged, resulting in sheaths inappropriately thick for the new (reduced) fiber caliber (28). We found that the maximum number of lamellae was the same ( $\sim 130$ ) in regenerating fibers with reduced  $\phi$  values and in control fibers with

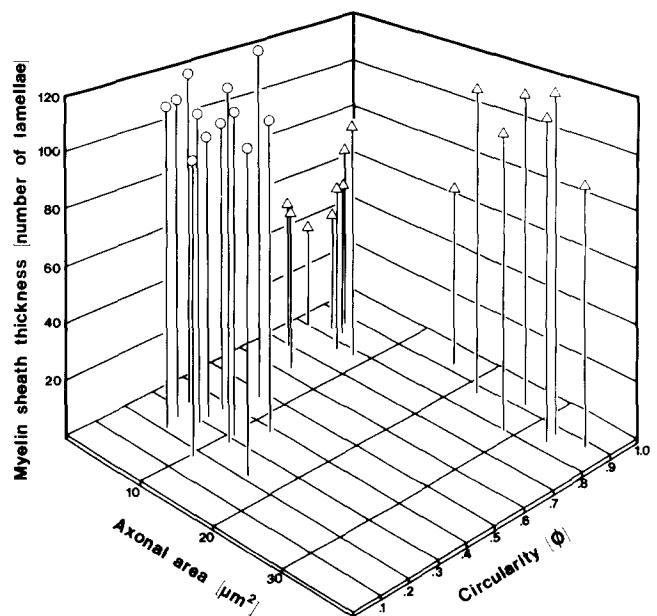


FIGURE 9 Myelin sheath thickness and axonal circularity. The relationship between myelin sheath thickness and axonal area is compared in axons that differ in their circularity. Myelin sheath thickness is expressed as the number of myelin lamellae per fiber. Circularity is measured using the parameter  $\phi$  (Fig. 8). Myelin sheath thickness is compared in regenerating axons (O) with low  $\phi$  values ( $<0.5$ ) and control axons ( $\Delta$ ) having similar areas (up to  $15 \mu\text{m}^2$ ) but normal  $\phi$  values ( $>0.6$ ). Myelin sheath thickness is also examined in larger control axons ( $15\text{--}40 \mu\text{m}^2$ ) with normal  $\phi$  values.

normal  $\phi$  values (Fig. 9). Because regenerating axons with reduced  $\phi$  values had significantly thicker myelin sheaths than control axons of equal area (with normal  $\phi$  values) (Fig. 9), these results indicated that these regenerating axons had undergone reductions in caliber. Thus, regenerating roots appeared to contain two populations of axons—those with reduced  $\phi$  values that had undergone reductions in caliber and those with normal  $\phi$  values in which caliber was unchanged. This distinction is important when selecting axons in which to demonstrate that reductions in area were associated with proportional changes in neurofilament number. Clearly, this correlation cannot be made unless we can identify axons that have undergone reductions in caliber.

#### *Loss of Caliber Is Associated with Reductions in Neurofilament Number*

To determine whether reductions in axonal area were associated with proportional decreases in neurofilament content, the relationship between axonal area and neurofilament number was examined in regenerating and control axons (Fig. 10). The total numbers of microtubule and neurofilament cross sections per axon, the total area of the axon, and the total area occupied by membranous structures (i.e., vesicles, mitochondria, and agranular reticulum) were measured in each axon. The axons examined in this study were located at proximal levels of the roots from the regenerating and contralateral control nerves of an animal killed 3 wk after nerve crush. Regenerating axons with low  $\phi$  values, i.e., those which had undergone reductions in caliber, were chosen for this study. This included the largest axons in the regenerating root (i.e., those with areas of  $35 \mu\text{m}^2$ ) as well as smaller ones (5–

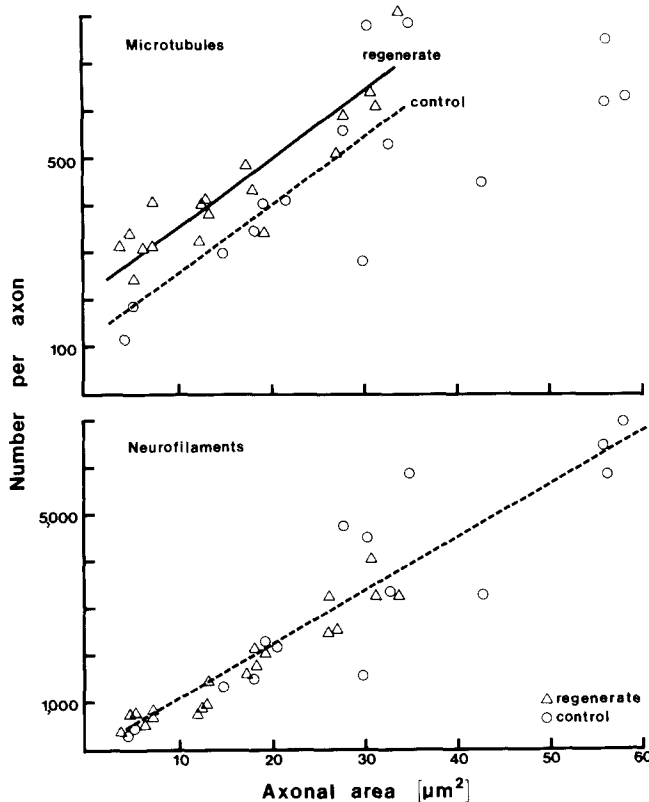


FIGURE 10 Changes in the number of neurofilaments and microtubules in regenerating axons. The number of microtubules (upper profile) and neurofilaments (lower profile) in regenerating ( $\Delta$ ) and contralateral control ( $\circ$ ) axons at the proximal level of the L5 ventral root 3 wk after sciatic nerve crush are plotted as a function of axonal area. Regenerating axons with low  $\phi$  values ( $<0.5$ ), i.e., those that had undergone reductions in caliber, were selected for this analysis. Analysis of co-variance revealed an identical linear relationship between axonal area and neurofilament number in regenerating and control axons (broken line, lower profile). Regenerating axons (solid line) contained significantly more microtubules than control axons (broken line) of comparable size (upper profile).

$20 \mu\text{m}^2$ ). In the control root, both the largest axons, i.e., with areas of  $60 \mu\text{m}^2$ , as well as those with areas comparable to regenerating axons were analyzed.

The proportion of axonal area occupied by membranous structures and mitochondria was equal in regenerating and control fibers ( $1.34 \pm 0.22\%$  and  $1.11 \pm 0.07\%$ , respectively). Analysis of covariance revealed that the number of neurofilament cross sections per fiber was the same in regenerating and control axons of equal area. In contrast, the number of microtubules per fiber was greater in regenerating axons than in control axons of equal area (Fig. 10). In both regenerating and control axons, the relationship between neurofilament number and axonal area was best described by a single straight line;  $y = 114.71x - 77.89$ , where  $y$  equals the number of neurofilaments and  $x$  equals axonal area in square millimicrons. Thus, the density of neurofilaments was equal in regenerating and control axons having the same area. For example, in regenerating and control axons with areas of  $10 \mu\text{m}^2$ , the density of neurofilaments was  $107/\mu\text{m}^2$ . The number of microtubules in control axons increased linearly with area for axons  $<30 \mu\text{m}^2$ . With further increase in area, the number remained fairly constant (Fig. 10). Over the entire range of

area, i.e.,  $5\text{--}60 \mu\text{m}^2$ , the relationship between the number of microtubules and the area in control axons was best described by the equation  $y = -0.22x^2 + 23.61x + 31.07$ , where  $y$  equals the number of microtubules and  $x$  equals area in square millimicrons. For axons with areas  $<30 \mu\text{m}^2$ , this relationship was best described by straight lines in both regenerating and control axons. These lines were parallel, i.e., they had the same slopes but different intercepts (Fig. 10, upper profile). For regenerating axons,  $y = 14.73x + 207.09$ , whereas for controls  $y = 14.73x + 110.69$ . Thus, for axons of equal area, regenerates contained significantly more microtubules than controls. For example, in axons with areas of  $10 \mu\text{m}^2$ , the density of microtubules in regenerates ( $35/\mu\text{m}^2$ ) was significantly greater than in controls ( $26/\mu\text{m}^2$ ). From these results, we conclude that reductions in the area of regenerating axons were associated with a proportional decrease in the number of neurofilaments.

## DISCUSSION

### *Neurofilaments as Intrinsic Determinants of Caliber in Myelinated Axons*

Our results indicate that neurofilament content is a major determinant of axonal caliber in myelinated fibers. Cytoskeletal elements and their surrounding domains accounted for nearly all ( $>98\%$ ) of the cross-sectional area of myelinated axons in both regenerating and control roots. Neurofilaments were the principal cytoskeletal structures in these axons, where they outnumbered microtubules by as much as 10:1. Regenerating axons, which showed reductions in caliber, were identified on the basis of reduced circularity. In these axons, neurofilament density (number/ $\mu\text{m}^2$ ) was identical to that in area-matched controls. In contrast, microtubule density was higher in regenerating axons than in controls. Although membranous structures (mitochondria, vesicles, and agranular reticulum) occupied an equal proportion of the cross-sectional area of regenerating and control axons, this area represented a relatively minor part of the total ( $<2\%$ ). Thus, reduced cross-sectional area in regenerating axons was correlated with a proportional decrease in neurofilament number.

Three additional lines of evidence indicate that neurofilament content is a primary determinant of axonal caliber: ultrastructural examination of cytoskeletal composition among fibers differing in caliber; morphometry of different regions along the course of individual fibers; and examination of normal developing axons. The first approach was used by Friede and Samorajski (9), who quantitatively compared cytoskeletal components among axons of different calibers. Small axons had a high proportion of microtubules with relatively few neurofilaments. Among larger fibers, neurofilament number was the major correlate of axonal caliber. Although the numbers of both neurofilaments and microtubules were directly correlated with caliber, neurofilaments greatly outnumbered microtubules in large-caliber axons (by as much as 10:1). The present study revealed a similar relationship between neurofilament number and cross-sectional area: microtubule numbers were related in a nonlinear fashion with little increase in microtubule number in axons with cross-sectional areas  $>30 \mu\text{m}^2$ .

The second approach, assessment of cytoskeletal composition along different regions of single axons, provides a dramatic illustration of the relationship between neurofilament

number and axonal diameter. Large myelinated fibers in the peripheral nervous system have periodic variations in axonal caliber; in the region around nodes of Ranvier, there is a segment of reduced caliber. This constricted segment, composed of myelin sheath attachment sites in the paranodes and the node of Ranvier, may have a cross-sectional area as little as one-tenth that of the adjacent internodes. Using skip-serial electron micrographs of individual large myelinated fibers to assess the numbers and densities of neurofilaments and microtubules in internodes and in constricted segments, Berridge (11) found that the number of neurofilaments/ $\mu\text{m}^2$  (i.e., neurofilament density) was similar in nodal and internodal regions. In other words, neurofilament number was markedly reduced in regions of smaller caliber, and this reduction directly correlated with axonal area. In contrast, microtubule number was essentially the same in internodal regions and in constricted segments; the density was, thereby, greatly increased in the constricted segments. Consequently, the ratio of neurofilaments to microtubules was much greater in the internodes than in the nodes.

The third line of evidence relating neurofilament content to caliber comes from studies of normal axonal development. Immature axons in the peripheral nervous system and central nervous system are composed largely of microtubules with relatively few neurofilaments (29). Growth in caliber is associated with an increase in neurofilament content of the axon. Microtubule numbers also increase as the axon grows in caliber but at a much slower rate than neurofilament numbers.

The density of neurofilaments ( $\sim 100/\mu\text{m}^2$ ) was remarkably similar in axons with widely differing calibers. The relatively regular spacing between neurofilaments (10, 30) apparently reflects the presence of interfilament cross-links (31–33). Recent evidence indicates that the 200-kilodalton neurofilament protein is associated with these cross-links (3, 34). Thus, cross-link formation may be an intrinsic property of neurofilaments which regulates their spacing and, thereby, determines the volume which they occupy.

### *Changes in Axonal Caliber Reflect Alterations in Neurofilament Transport*

The number of neurofilaments was reduced in the proximal stumps of regenerating fibers. Inasmuch as essentially all of the neurofilament protein in the axon appears to be in its polymeric (filamentous) form (7), this reduction in neurofilament number is consistent with the recent demonstration that there is a decrease in the amount of neurofilament protein in the proximal stump (35). In addition, because neurofilament protein is synthesized exclusively in the cell body and because axonal neurofilaments appear to be continuously transported in a proximal-to-distal direction, the neurofilament content of the axon should reflect the quantity of neurofilament protein entering the axon from the cell body. Therefore, this reduction in neurofilament content of the proximal stump could result from a decrease in the quantity of neurofilaments transported into these axons. This is supported by our previous observation that the amount of labeled neurofilament protein transported in the proximal stump of regenerating fibers (20 d after axotomy) is selectively reduced in comparison to tubulin and actin (14). In fact, this relative decline in neurofilament transport could account for the observed reduction in the ratio of neurofilaments to microtubules in these regenerating axons.

Further support for the role of neurofilament transport in this process comes from our recent observation that changes in transport are correlated with alterations in caliber throughout the entire course of the regenerative response. The relative amount of labeled neurofilament protein transported in lumbar motor neurons is reduced throughout the period when caliber is decreased, i.e., up to and including 6 wk after axotomy. Recovery of caliber is associated with an increase in neurofilament transport to above-normal levels (Hoffman, P. N., G. W. Thompson, J. W. Griffin, and D. L. Price, unpublished observations).

Although, normally, there appears to be relatively little turnover of neurofilament protein within the axon (before reaching axon terminals) (12), an increase in turnover during regeneration could account for the reduced neurofilament content of the axon and the relative decrease in the amount of neurofilament protein transported in the slow component. The principal evidence against this possibility (increased turnover) and in favor of reduced neurofilament transport is the observation that reductions in caliber associated with these decreases in neurofilament content proceed along the proximal stump in a proximal-to-distal direction at a rate equal to the velocity of neurofilament transport (Fig. 11). It is conceivable that a mediator of turnover, e.g., an activator of the protease or the activated protease itself, is slowly transported within these axons, thereby accounting for the proximal-to-distal progression of these changes. However, it is much more likely that these changes in neurofilament content reflect the reduced transport of neurofilaments from the cell body.

Sinicropi and McIlwain (35) used two-dimensional gel electrophoresis to measure the amount of neurofilament protein in cell bodies isolated from the lumbar spinal cord of frogs 20 d after crushing the sciatic nerve. They found that cell bodies of regenerating motor neurons contained significantly more neurofilament protein than those of controls. Such an increase in perikaryal neurofilament content could reflect impaired entry of neurofilaments into the axon. However, it is unlikely that this is the sole mechanism for reduced neurofilament transport. If there were no concomitant reduction in neurofilament synthesis, then neurofilaments normally destined to enter the axon would rapidly accumulate in the cell body, assuming that their turnover in the cell body was not increased. This would lead to the formation of giant neurofilament-rich masses in the cell body similar to those observed

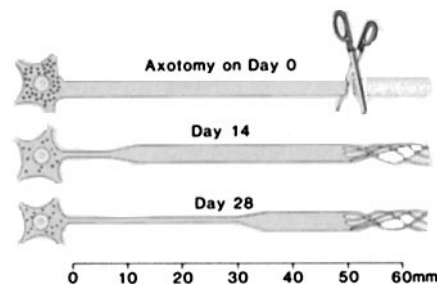


FIGURE 11 Schematic illustration of the spatial and temporal evolution of reduction in caliber in the proximal stump of a transected nerve fiber. To simplify this diagram, myelin sheaths and nodes of Ranvier are not shown. Reductions in caliber first appeared proximally and then spread along the axon in a proximal-to-distal direction at a rate equal to the velocity of neurofilament transport (i.e., 1.7 mm/d in lumbar motor neurons of rat). The morphological changes associated with chromatolysis were not prominent in these neurons.



in the proximal axon after systemic intoxication with  $\beta,\beta'$ -iminodipropionitrile, an agent which selectively impairs axonal transport without major changes in the synthesis of neurofilaments (36, 37). Within several weeks after intoxication with  $\beta,\beta'$ -iminodipropionitrile, these swellings often attain diameters ( $>100\ \mu\text{m}$ ) several times greater than that of the cell body. Although the volume of the cell body may increase somewhat during regeneration (35), changes of this magnitude have not been observed. This suggests that reductions in neurofilament transport result from a decrease in synthesis. Perhaps increased levels of neurofilament protein in the cell body, resulting from decreased delivery to the axon, lead to reductions in neurofilament synthesis. Such a mechanism appears to be responsible for reductions in the synthesis of tubulin after the levels of unassembled tubulin in the cell are experimentally increased by agents which block polymerization, e.g., colchicine (38).

### Neuron-Target Cell Interactions

Fiber caliber is the principal determinant of impulse conduction velocity along the axon (39). In this way, caliber strongly influences interactions between the neuron and its target. Conversely, neuron-target cell interactions also appear to play an important role in the control and maintenance of caliber. This is demonstrated dramatically after axotomy, where disconnection of the neuron and its targets leads to atrophy of the proximal stump. Our results are consistent with those of previous studies, which found that failure of regeneration and/or reconnection results in persistent atrophy, whereas recovery coincides with reconnection (26, 40). The results of our studies suggest that interactions between the neuron and its targets may regulate the expression of a major class of neuron-specific genes (i.e., those coding for the neurofilament proteins). By regulating the level of neurofilament production and transport, these interactions play a major role in determining axonal form (caliber) and function (impulse-conduction velocity). Our results indicate that changes in axonal caliber represent a quantifiable response to axotomy which should eventually allow investigation of the signals responsible for these alterations in neurofilament synthesis.

### Summary

The axon is uniquely suited for examining the role of the cytoskeleton in the control of cellular volume. By taking advantage of the previous observations that the axonal transport of neurofilament protein is selectively reduced in comparison to other cytoskeletal proteins in regenerating neurons (14), we have demonstrated that neurofilaments, the intermediate filaments of nerve cells, are major intrinsic determinants of axonal caliber, and that the axonal transport of these structures plays an important role in regulating the caliber of individual fibers. These results are consistent with the suggestion that intermediate filaments may play a general role in the control of cellular volume.

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