

Changes in Neurofilament Transport Coincide Temporally with Alterations in the Caliber of Axons in Regenerating Motor Fibers

PAUL N. HOFFMAN,* GARY W. THOMPSON,† JOHN W. GRIFFIN,§ and DONALD L. PRICE¶

**Laboratory of Neuropathology and The Wilmer Ophthalmological Institute, †Laboratory of Neuropathology, §Departments of Neurology and Neuroscience, and ¶Departments of Neurology, Pathology, and Neuroscience, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205*

ABSTRACT The delivery of neurofilaments via axonal transport has been proposed as an important mechanism for regulating axonal caliber. If this hypothesis is correct, alterations in axonal caliber should appear coincident with changes in the delivery of neurofilaments to the axon. The purpose of this study was to determine whether alterations in the caliber of axons in the proximal stumps of transected motor fibers precede, coincide with, or occur substantially later than changes in the delivery of neurofilaments via axonal transport.

Between 3 d and 12 wk after crushing the sciatic nerves of 7-wk-old rats, lumbar motor neurons were labeled by the intraspinal injection of [³⁵S]methionine. In neurons labeled between 3 d and 6 wk after axotomy, the relative amount of neurofilament protein in the slow component, as reflected by the ratio of the radioactivities of the 145-kD neurofilament protein to tubulin, was reduced to 30–40% of the control value. Moreover, as determined by immunoreactivity on blots, the amounts of neurofilament protein and tubulin in these nerve fibers were reduced fourfold and twofold, respectively. Thus, changes in the ratio of labeled neurofilament protein to tubulin correlated with comparable changes in the quantities of these proteins in nerve fibers. This decrease in the quantity of neurofilament proteins delivered to axons coincided temporally with reductions in axonal caliber. After regeneration occurred, the delivery of neurofilament proteins returned to pre-axotomy levels (i.e., 8 wk after axotomy), and caliber was restored with resumption of normal age-related radial growth of these axons. Thus, changes in axonal caliber coincided temporally with alterations in the delivery of neurofilament proteins. These results suggest that the majority of neurofilaments in these motor fibers continuously move in the anterograde direction as part of the slow component of axonal transport and that the transport of neurofilaments plays an important role in regulating the caliber of these axons.

The caliber of an axon is a basic aspect of neuronal structure that correlates in general with perikaryal volume (1), myelin sheath parameters (2), excitability (3), and impulse conduction velocity (4). Although the determinants of axonal caliber are poorly understood, the number of neurofilaments within the axon correlates closely with axonal cross-sectional area (5, 6). Reduced amounts of neurofilament proteins are transported in the proximal stumps of transected motor fibers (7).

Axonal caliber is also reduced in the proximal stumps (8–16). This reduction in caliber, which correlates with a proportional decrease in axonal neurofilament content (8, 16), starts proximally and proceeds anterograde along nerve fibers at a rate equal to the velocity of neurofilament transport (8).

Based on correlative morphometric and axonal transport studies of these regenerating nerves and of developing nerves, we recently proposed that neurofilament content is an impor-

tant intrinsic determinant of caliber for myelinated axons, and that caliber can be changed by alterations in the amount of neurofilament protein entering the axon (8), or by alterations in the velocity of the axonal transport of neurofilaments (17). These hypotheses assume that most of the neurofilaments in the cytoskeleton are not stationary (i.e., undergo anterograde transport). In addition, they predict that changes in the amount of neurofilament proteins delivered to a given region of the axon should produce coincident changes in axonal caliber within that segment. If these predictions are not supported by studies of the precise timing of changes in caliber and neurofilament transport, then the relationship between neurofilaments and caliber and our conception of the dynamic organization of the cytoskeleton would require substantially different interpretations.

The purpose of this study was to determine whether changes in the axonal transport of neurofilaments precede, coincide with, or occur after alterations in the caliber of axons in the proximal stumps of transected motor fibers. The observation that changes in the delivery of neurofilaments precede alterations in caliber would suggest that most of the neurofilaments in the cytoskeleton are stationary (i.e., the moving fraction occupies a relatively small proportion of axonal cross-sectional area, thereby contributing little to the control of caliber). Under these circumstances, reductions in caliber would result from the turnover of stationary neurofilaments. Demonstration of coincident changes in the delivery of neurofilament proteins and alterations in caliber would support two conclusions: first, that neurofilament transport plays an important role in determining axonal caliber; and second, that the majority of neurofilaments in the cytoskeleton are transported in the anterograde direction by slow axonal transport (i.e., are moving rather than stationary). Finally, if changes in delivery occur after alterations in caliber, then changes in neurofilament transport could be secondary to changes in caliber (e.g., alterations in some other component of the cytoskeleton could be primarily responsible for reductions in caliber with changes in neurofilament transport occurring later).

MATERIALS AND METHODS

Experimental Procedures: Male Sprague-Dawley rats of ages 5–18 wk were used in these studies. Surgical procedures were carried out using chloral hydrate anesthesia (400 mg/kg, i.p.). Sciatic nerves in 7-wk-old animals (one nerve in each animal) were crushed at the junction of the L4 and L5 spinal roots using watch-maker's forceps.

At 3 d and 1, 2, 3, 4, 5, 6, 8, 10, and 12 wk after axotomy, lumbar motor neurons were labeled by the intraspinal injection of microliter quantities of [³⁵S]methionine (1,200 Ci/mM) (Amersham Corp., Arlington Heights, IL) using our published procedure (18). Isotope was injected at a final concentration of 80 mCi/ml. Control motor neurons were labeled in unoperated 8-, 12-, and 18-wk-old animals. In most cases, at least three animals were labeled in each experimental and control group.

Analyzing the Distribution of Labeled Proteins: Animals were killed 5 d after labeling. To study the distribution of labeled neurofilament proteins and tubulin along sciatic nerves, we homogenized 3-mm nerve segments in a solution containing 5% SDS, 8 M urea, and 5% 2-mercaptoethanol and analyzed the labeled proteins using SDS PAGE and gel fluorography (7). Exposure times for fluorograms were calculated so that the product of radioactivity in the most heavily labeled segment (in cpm) and the exposure period (in days) equaled 1.5×10^6 cpm days. After the fluorograms were developed, the bands corresponding to the neurofilament proteins and tubulin were removed from the gels, dissolved in 30% H₂O₂, and levels of radioactivity measured using liquid scintillation spectroscopy.

Since the 145-kD neurofilament protein is more completely separated from other labeled gel bands than either the 200- or 68-kD proteins, it was used for

determining the distribution of neurofilament-associated radioactivity along the nerve (19). Levels of radioactivity in the 145-kD protein were normalized in relation to radioactivity in tubulin using the ratio of the sum of radioactivity along the nerve in the 145-kD protein to the sum of radioactivity in tubulin.

Quantitation of Neurofilament Protein and Tubulin in Regenerating and Control Ventral Roots: Immunoreactivity on blots was used to compare the relative quantities of neurofilament protein and tubulin in the roots of regenerating and control nerves 2 wk after crushing the sciatic nerve. Control roots were obtained from unoperated, age-matched animals. 5-mm segments of L5 ventral roots (extending 0–5 mm from the spinal cord) were homogenized in 700 μ l of a solution containing 8 M urea, 5% 2-mercaptoethanol, and 50 mM sodium phosphate, pH 6.5. Homogenates were incubated in a boiling water bath for 4 min. Aliquots (40 μ l) of the original homogenate and of serial dilutions of the homogenate (i.e., 1:2, 1:4, 1:6, and 1:8) were dotted onto nitrocellulose membranes using a Hybri-Dot manifold (Bethesda Research Laboratories, Inc., Gaithersburg, MD). Immunostaining with PAP-immunoperoxidase was carried out according to the method of Goldstein et al. (20) using monoclonal antibodies to the 200-kD neurofilament protein (06–17 and 02–40) (provided by Drs. Nancy and Ludwig Sternberger) and a monoclonal antibody to beta tubulin (obtained from Amersham Corp.). The intensity of immunostaining was proportional to the amount of protein in each aliquot (i.e., it declined with increasing dilution). Therefore, the relative amounts of neurofilament protein and tubulin in regenerating and control roots could be estimated by finding the dilutions that gave comparable levels of immunostaining as determined both visually and using an LKB 2202 Ultrascan laser densitometer (LKB Produkter AB, Bromma, Sweden). Proteins in these roots were also analyzed on SDS gels, transferred electrophoretically to nitrocellulose paper, and immunostained with these antibodies (Western blots). Each antibody recognized a single gel band; the intensity of staining was proportional to the amount of protein loaded.

Preparation of Tissue for Morphometric Analysis: At 1, 2, 3, 4, 5, 6, 8, and 10 wk after axotomy, animals were perfused through the ascending aorta with 0.9% saline followed by 5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.3 (8). Unoperated control animals were perfused at 5, 8, 12, and 18 wk of age. Three animals were perfused in each group except for a single control animal at 18 wk of age. After the animals were stored at 4°C for 18 h, the L5 ventral roots were removed and thoroughly rinsed in 0.1 M phosphate buffer, pH 7.3. 3-mm segments obtained from the proximal level of the roots (i.e., adjacent to the spinal cord) were postfixed in osmium tetroxide, dehydrated in graded alcohol, and embedded in Epon. Transverse sections (1- μ m thick) were stained with toluidine blue and examined using light microscopy. To study very early changes in caliber near motor neuron cell bodies, spinal cords were sectioned longitudinally in a plane parallel to the ventral surface (i.e., motor axons were sectioned transversely along the segments extending from the cell body to the root exit zone).

Measurement of Axonal Caliber: The cross-sectional areas of axons at the proximal level of the L5 ventral root were measured in animals killed at weekly intervals after axotomy and in 5-, 8-, 12-, and 18-wk-old control animals using our published method (8). Axonal diameters were calculated from the measured areas. At least 200 axons were analyzed in each root; three roots were analyzed at each time after axotomy and in each control group (except at 18 wk of age). The mean areas of the largest 25% of axons were determined in each experimental and control group.

RESULTS

Changes in the Distribution of Labeled Proteins in the Proximal Stumps of Transected Nerve Fibers

After injection of [³⁵S]methionine into the lumbar spinal cord, labeled neurofilament triplet proteins (200, 145, and 68 kD), tubulin (55 kD), and actin (43 kD) were transported distally along motor fibers (Fig. 1). The triplet proteins, which had coincident distributions along the nerve, extended farther distally in the proximal stumps of transected motor fibers than in comparable regions of control fibers. Actin and tubulin also extended farther distally along regenerating nerves than did controls. In addition, several other polypeptides, located in the same region of the nerve as tubulin and actin, were more prominently labeled in regenerating fibers than in controls (Fig. 1).

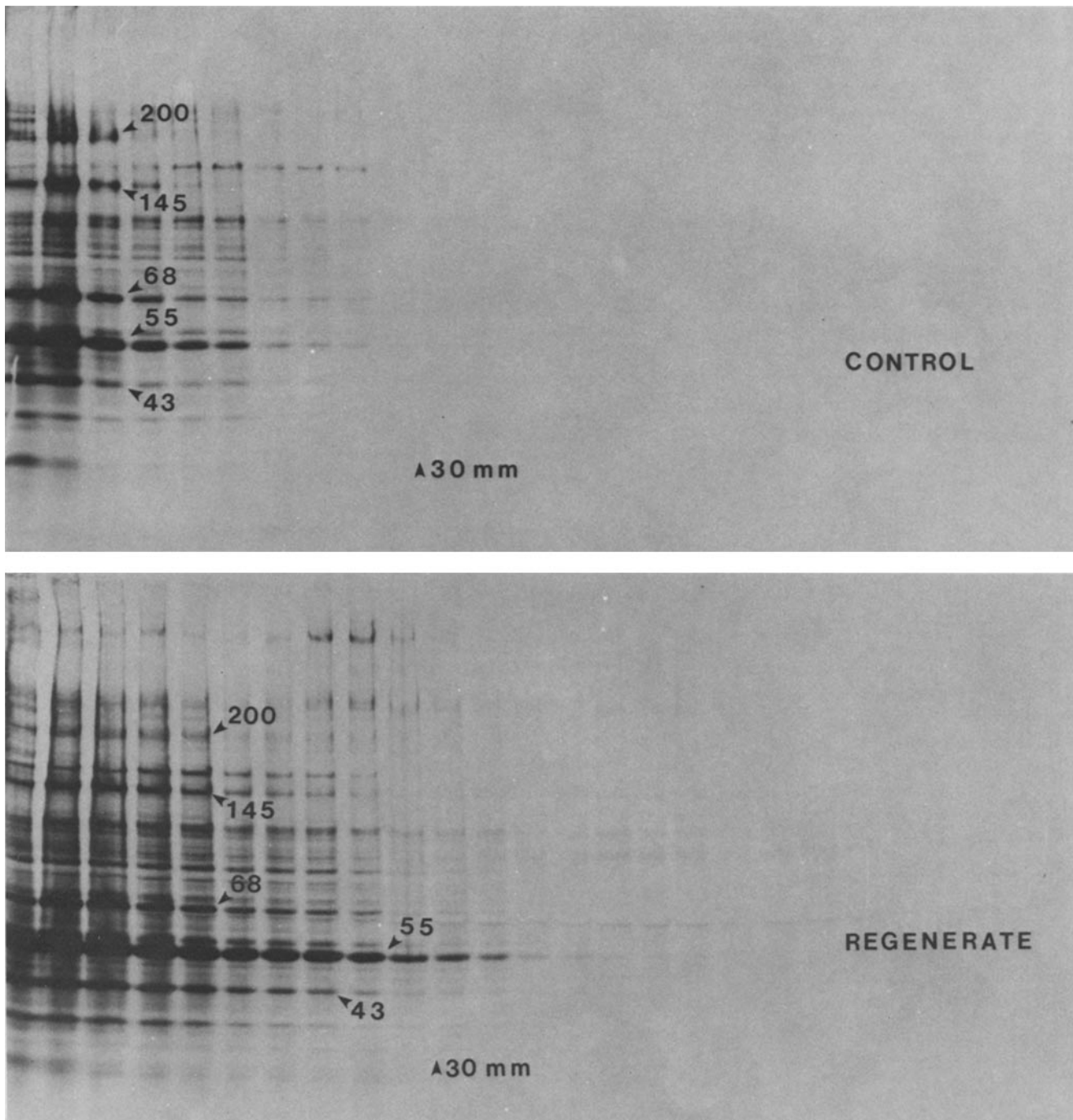


FIGURE 1 Axonal transport of cytoskeletal proteins in regenerating and control motor fibers. These fluorograms of gradient SDS slab gels (5–17% acrylamide) illustrate the distribution of the neurofilament triplet proteins (200, 145, and 68 kD), tubulin (55 kD), and actin (43 kD) along the L5 ventral roots and sciatic nerves 5 d after lumbar motor neurons were labeled by the intraspinal injection of [³⁵S]methionine. Regenerating neurons were labeled 3 wk after crushing the sciatic nerve (lower gel), while control neurons were labeled in an unoperated 8-wk-old animal (upper gel). Each gel slot contains labeled proteins from a 3-mm nerve segment. Distance along the nerve (i.e., at 30 mm) is indicated at the bottom. The highest levels of radioactivity in segments of the regenerating and control nerves were 150,000 and 83,000 cpm, respectively; the fluorograms were exposed for 10 and 18 d, respectively.

Changes in the distribution of neurofilament-associated radioactivity along the nerve were examined in detail for the 145-kD neurofilament protein. The normally well-defined wave of radioactivity in this protein broadened to extend farther distally along regenerating fibers than controls in neurons labeled between 3 d and 6 wk after axotomy (Figs.

2–4). The shape of this curve returned to normal in neurons labeled 8 wk or more after axotomy (Fig. 4).

Radioactivity in tubulin was also distributed along control nerves in a well-defined wave (Fig. 2). In neurons labeled between 3 d and 2 wk after axotomy, this wave broadened and extended farther distally along the nerve (Fig. 2). In

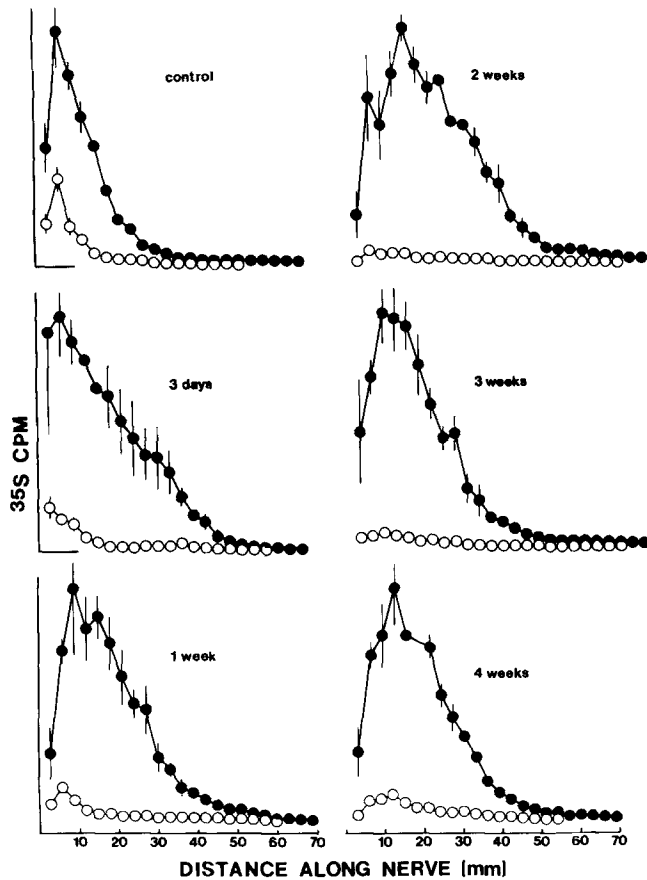


FIGURE 2 Reduced axonal transport of neurofilament proteins in regenerating motor fibers. The distributions of radioactivity in the 145-kD neurofilament protein (○) and tubulin (●) along the proximal stumps of transected motor fibers and along the same region of control motor fibers is examined 5 d after lumbar motor neurons were labeled by the intraspinal injection of [³⁵S]methionine. Regenerating neurons were labeled 1–4 wk after the sciatic nerves were crushed 50 mm from the spinal cord. Control neurons were labeled in unoperated 8-wk-old animals. Mean levels of radioactivity in the 145-kD neurofilament protein are normalized in relation to radioactivity in tubulin. Each data point represents the mean of six values for the control nerves; four values each at 3 d, 1 wk, and 3 wk; three values at 2 wk; and two values at 4 wk. Vertical bars indicate the standard errors of the means except at 4 wk where the bar indicates the range of values. The highest points in these profiles for tubulin and the 145-kD neurofilament protein, respectively, are: 3,134 and 820 cpm for controls; 5,394 and 1,360 cpm at 3 d; 8,597 and 969 cpm at 1 wk; 7,507 and 709 cpm at 2 wk; 9,924 and 959 cpm at 3 wk; and 8,360 and 1,465 cpm at 4 wk.

neurons labeled 3 wk after axotomy, the tubulin wave began to decrease in width (Fig. 2). In neurons labeled 8 or more weeks after axotomy, the wave showed its original shape (Fig. 4).

Changes in the Levels of Radioactivity in Transported Neurofilament Proteins

Compared with tubulin, labeling of the 145-kD neurofilament protein was reduced in neurons isolated between 3 d and 6 wk after axotomy (Figs. 1, 2, and 4). The ratio of the radioactivities of the 145-kD neurofilament protein to tubulin was reduced to 30–40% of the control value in neurons labeled between 3 d and 6 wk after axotomy; this ratio returned to

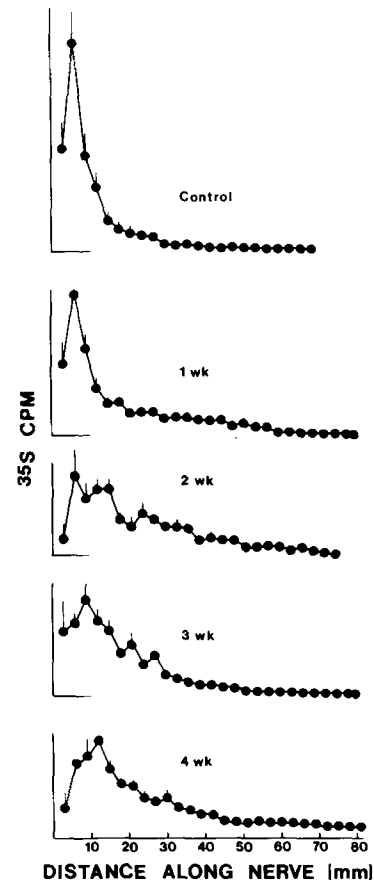


FIGURE 3 Changes in the shape of the neurofilament distribution in regenerating motor fibers. The distributions of radioactivity in the 145-kD neurofilament protein along the proximal stumps of transected motor fibers and along corresponding regions of control fibers is examined 5 d after lumbar motor neurons were labeled by the intraspinal injection of [³⁵S]methionine. Regenerating motor neurons were labeled 1–4 wk after axotomy. Control neurons were labeled in unoperated 8-wk-old animals. Each data point represents the mean of six values in control nerves, four values each at 1 and 3 wk, three values at 2 wk, and two values at 4 wk. The levels of radioactivity in these profiles were normalized to facilitate their comparison. For actual levels of radioactivity see Fig. 2. Vertical bars indicate the standard errors of the means except at 4 wk where the bar indicates the range of values.

control levels in neurons labeled 8 wk or more after axotomy (Fig. 5). Since the relative amounts of radioactivity in each of the neurofilament proteins remained constant after axotomy (Table I), labeling of the 145- and 68-kD neurofilament proteins was reduced by the same degree (in relation to tubulin).

Reductions in the Amounts of Neurofilament Protein and Tubulin in the Proximal Stumps of Transected Motor Fibers

The immunoreactivities of tubulin and the 200-kD neurofilament protein were reduced in regenerating roots 2 wk after axotomy as compared with controls. Tubulin immunoreactivity was reduced twofold (Fig. 6). Immunoreactivity of the 200-kD neurofilament protein was reduced fourfold (Fig. 6); this reduction was observed using monoclonal antibodies against phosphorylated and nonphosphorylated epitopes of

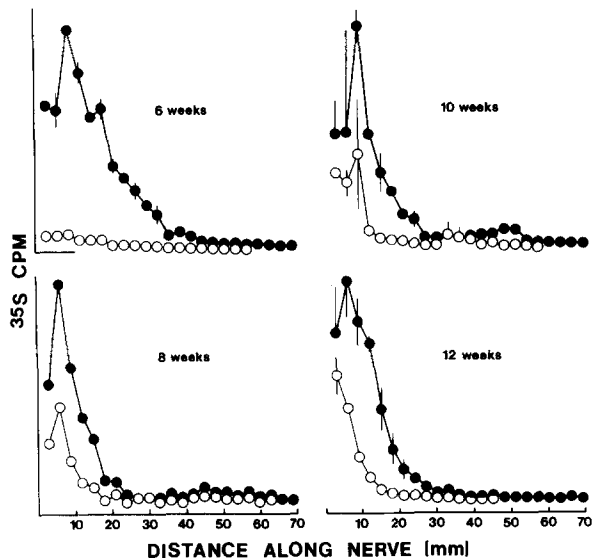


FIGURE 4 Recovery of neurofilament transport in regenerating motor fibers. The distributions of radioactivity in the 145-kD neurofilament protein (○) and tubulin (●) along the proximal stumps of transected motor fibers is examined 5 d after lumbar motor neurons were labeled by the intraspinal injection of [³⁵S]methionine. Regenerating neurons were labeled 6, 8, 10, and 12 wk after axotomy. The mean levels of radioactivity in the 145-kD neurofilament protein are normalized in relation to the total amounts of radioactivity in tubulin. Each data point represents the mean of two values at 6, 8, and 10 wk after crush, and three values at 12 wk. Vertical bars indicate the range of values except at 12 wk where the bar indicates the standard error of the mean. The highest points in these profiles for tubulin and the 145-kD neurofilament protein, respectively, are: 7,546 and 1,796 cpm at 6 wk; 1,350 and 566 cpm at 8 wk; 2,106 and 909 cpm at 10 wk; and 12,634 and 5,089 cpm at 12 wk.

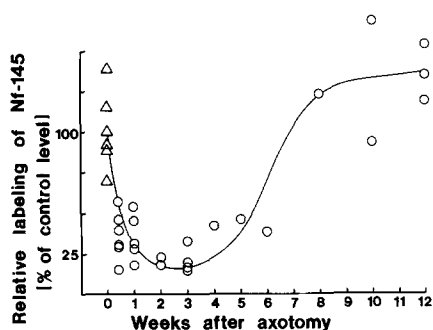


FIGURE 5 Changes in the relative amount of neurofilament protein transported in the slow component. We examined the ratio of 145-kD neurofilament protein to tubulin radioactivity in control nerves (Δ) and in the proximal stumps of regenerating nerves (○) in neurons labeled between 3 d and 12 wk after axotomy. These ratios were determined in individual sciatic nerves 5 d after lumbar motor neurons were labeled by the intraspinal injection of [³⁵S]methionine. Each data point represents the value in an individual nerve. These ratios were normalized in relation to the mean control value and plotted as a function of the time at which neurons were labeled.

the 200-kD protein (i.e., antibodies 06-17 and 02-40, respectively) (21). Immunoreactivity decreased with increasing dilution of the samples (i.e., immunoreactivity was proportional to protein concentration) (Fig. 6). Therefore, compared with controls, regenerating roots contained approximately fourfold less 200-kD neurofilament protein and twofold less tubulin.

TABLE I. Labeling of the Neurofilament Proteins at Various Times after Axotomy*

Time after Axotomy	Neurofilament proteins			Number of animals
	200 kD	145 kD	68 kD	
control [†]	14 ± 2	25 ± 5	60 ± 4	5
3 d	15 ± 4	24 ± 3	62 ± 4	6
1 wk	16 ± 4	25 ± 6	59 ± 9	6
2 wk	15 ± 2	24 ± 0	62 ± 2	2
3 wk	16 ± 3	23 ± 4	61 ± 6	4
4 wk	14	26	60	1

* Mean percentage of neurofilament-associated radioactivity ± standard deviation. Lumbar motor neurons were labeled by the intraspinal injection of [³⁵S]methionine at various times after crushing the sciatic nerve (time after axotomy). Proteins in the proximal stump were analyzed 5 d after labeling using SDS PAGE and gel fluorography. Levels of radioactivity in the 200-, 145-, and 68-kD neurofilament proteins were measured using liquid scintillation spectroscopy (see Materials and Methods). Control roots were obtained from unoperated, 8- and 12-wk-old animals.

[†] Given a ratio of 14: 25: 60 for radioactivity in the 200-, 145-, and 68-kD neurofilament proteins, respectively, the estimated molar ratio of these proteins in the slow component is 1.0: 2.6: 6.8. This estimate, which takes into account the methionine contents (34) and relative sizes of these polypeptides, is in agreement with previous estimates of the molar ratio of triplet proteins in neurofilaments purified from spinal cord (34, 35).

In addition, since the relative amounts of radioactivity in each of the neurofilament proteins remained constant after axotomy (Table I), reductions in the 200-kD protein were accompanied by comparable reductions in the amounts of 145- and 68-kD neurofilament proteins in regenerating roots.

Changes in Axonal Caliber

In control fibers, axonal cross-sectional area increased linearly (i.e., at a rate of 2.88 μm²/wk in the largest 25% of fibers) between 5 and 18 wk of age (Fig. 7). The earliest changes in axonal caliber were seen in the intraparenchymal portions of transected motor fibers at 4 d after axotomy (Fig. 8). 1 wk after crushing the sciatic nerve, reductions in axonal caliber appeared at the proximal level of the L5 ventral root (i.e., at a distance of 3–5 mm from motor neuron cell bodies) (Fig. 7), a change that coincided temporally with the delivery of reduced quantities of neurofilament proteins to axons (Fig. 9). Between 1 and 3 wk after axotomy, cross-sectional area decreased maximally (i.e., at a rate of 5.56 μm²/wk in the largest 25% of fibers). The resumption of radial growth in these fibers (i.e., 8 wk after axotomy) coincided temporally with the return of neurofilament labeling to control levels (Fig. 9).

DISCUSSION

Axonal Transport of Reduced Quantities of Neurofilament Proteins in Regenerating Motor Fibers

Several lines of evidence indicate that reduced amounts of neurofilament protein were transported in regenerating motor fibers. First, the ratio of the radioactivities of the 145-kD neurofilament protein to tubulin was less in regenerating motor fibers (i.e., in neurons labeled between 3 d and 6 wk after axotomy) than in controls. Since the relative amounts of radioactivity in each of the neurofilament proteins did not change after axotomy, labeling of the 200- and 68-kD proteins was reduced to the same degree. In this way regenerating motor axons differ from developing retinal ganglion cell (optic

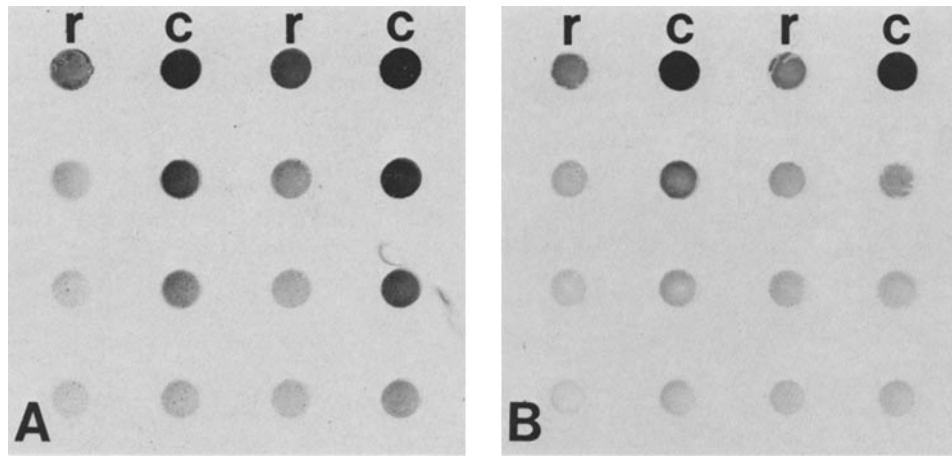


FIGURE 6 Estimates of the relative amounts of 200-kD neurofilament protein (A) and tubulin (B) in regenerating and control roots. Immunoreactivity is compared in corresponding regions of regenerating (r) and control (c) L5 ventral roots 2 wk after axotomy using monoclonal antibodies to the 200-kD neurofilament protein (02-40) and to beta tubulin. Specific antibody was detected on these nitrocellulose membranes using PAP-immunoperoxidase. Immunoreactivity, which is proportional to the amount of protein in each sample (i.e., it declines with increasing dilution of the samples), is fourfold less for neurofilament protein and twofold less for tubulin in regenerating roots than in controls. Samples from individual roots, in dilutions of 1:2, 1:4, 1:6, and 1:8 (from top to bottom) comprise each vertical column. These results are representative of those from 15 regenerating and 15 control roots.

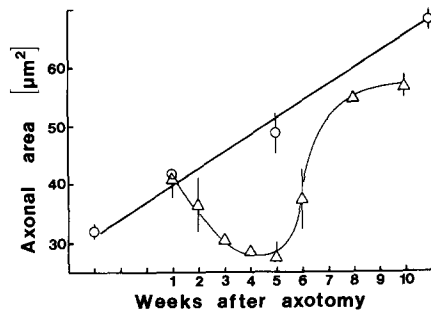


FIGURE 7 Changes in caliber in the proximal stumps of transected motor fibers. The mean cross-sectional areas of the largest 25% of axons were measured at the proximal level of the L5 ventral root in age-matched control animals (O) and in animals examined between 1 and 10 wk after axotomy (Δ). Standard errors of the means are indicated by vertical bars.

nerve) axons in neonatal rabbits, which contain less 200-kD neurofilament protein (in relation to the 145- and 68-kD proteins) (22). Second, regenerating roots contained lesser amounts of the three neurofilament proteins and tubulin than did control roots. The amounts of these four proteins have previously been reported to be decreased in the proximal stumps of transected motor fibers in frog (23). Thus, reductions in the ratio of the radioactivities of neurofilament protein to tubulin correlated with a reduction in the amount of neurofilament proteins in regenerating roots (i.e., rather than with an increase in tubulin and no reduction in neurofilament proteins). Taken together, these observations indicate that reduced amounts of neurofilament protein are transported in the proximal stumps of regenerating motor fibers. Since neurofilament proteins appear to be present almost entirely in their polymeric (filamentous) form in axons (24), these findings suggest that reduced numbers of neurofilaments are transported in regenerating nerve fibers. This is consistent with morphological evidence demonstrating reduced numbers of neurofilaments in the proximal stumps of regenerating motor fibers (8, 16).

The Role of Neurofilaments in the Control of Axonal Caliber

The major finding of this study is that changes in axonal caliber coincided temporally with alterations in the delivery of neurofilament proteins to the proximal stumps of transected motor fibers. These results have important implications for the role of neurofilaments in the control of axonal caliber and for the dynamic properties of neurofilaments in myelinated nerve fibers.

Several lines of evidence suggest that neurofilaments are major intrinsic determinants of axonal diameter in myelinated nerve fibers of mammals and other vertebrate species (1, 5, 6, 8, 25). Neurofilaments are the most numerous cytoskeletal elements in large-caliber axons (1, 5, 6, 8). In these axons, neurofilaments and their surrounding domains occupy most of the cross-sectional area (8, 26), and neurofilament number correlates linearly with axonal cross-sectional area (1, 5, 6, 8). Reduction in caliber in the proximal stumps of transected motor fibers correlates with a proportional decrease in the number of axonal neurofilaments (i.e., the density of neurofilaments remains constant) (8, 16). In contrast, microtubules do not appear to play an important role in regulating the diameters of these large-caliber motor fibers. In motor axons with cross-sectional areas greater than $30 \mu\text{m}^2$, increase in caliber is not correlated with a proportional increase in the number of microtubules (i.e., the number of microtubules remains constant) (1, 5, 8). In fact, regenerating motor fibers that have undergone reductions in caliber contain more microtubules than do area-matched control fibers (i.e., microtubule density increases, even though the total number of microtubules decreases as axonal caliber is reduced) (8).

Neurofilaments do not appear to play an important role in regulating axonal diameter in small-caliber nerve fibers (i.e., in unmyelinated axons, which generally attain diameters no greater than $1 \mu\text{m}$) (1, 27), or in the giant axons of some invertebrate species (e.g., crayfish) which lack neurofilaments and only contain microtubules (28). The major difference between the cytoskeletons of large- and small-caliber axons (of vertebrates) is their neurofilament content. Microtubules

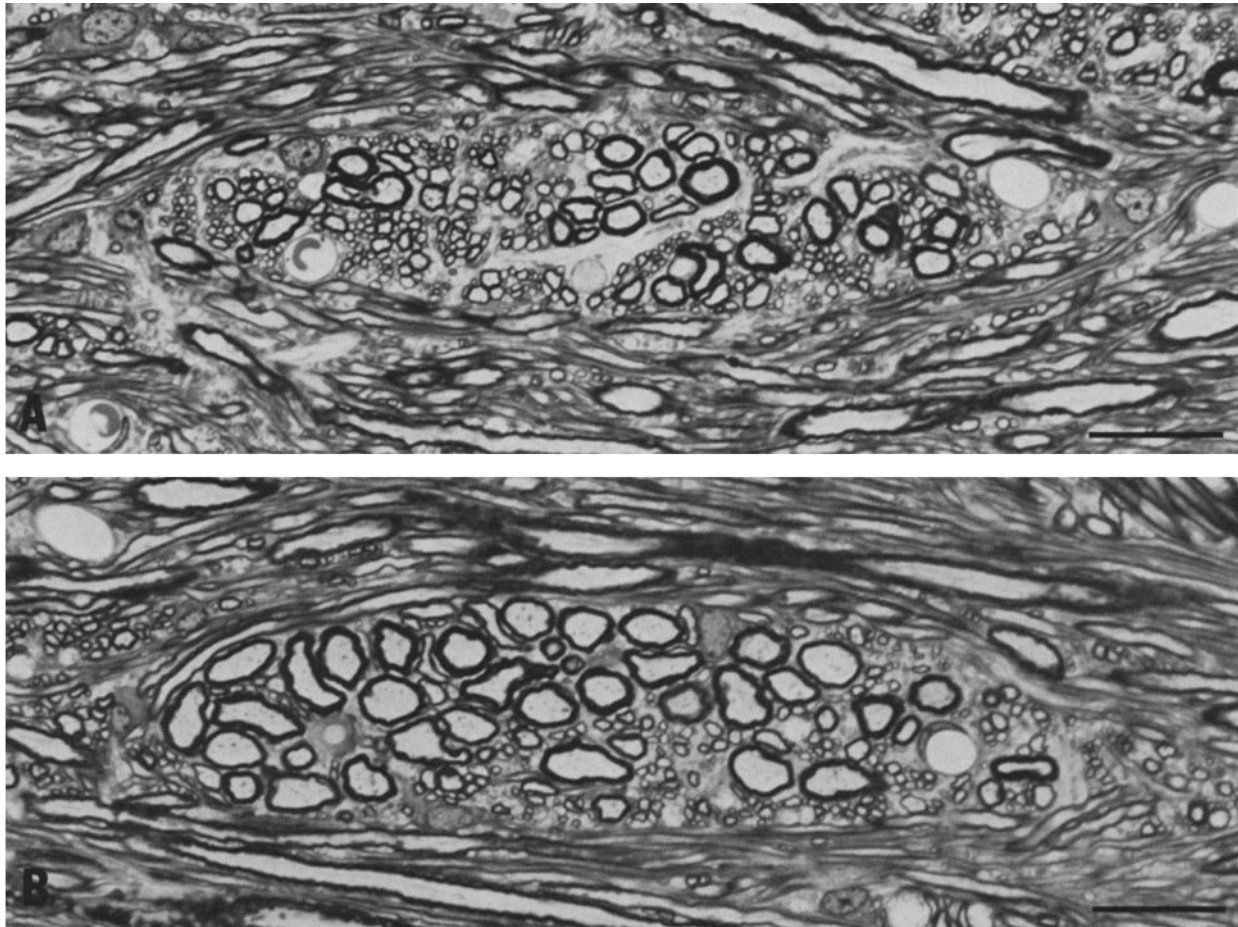


FIGURE 8 Reductions in axonal caliber in the intraparenchymal portions of motor fibers 4 d after axotomy. Transverse sections of the portions of motor fibers extending between the cell bodies and the root exit zone were obtained by sectioning the spinal cord longitudinally in a plane parallel to the ventral surface. Caliber is compared in axotomized (A) and in age-matched control fibers (B). Bars, 20 μm . $\times 880$.

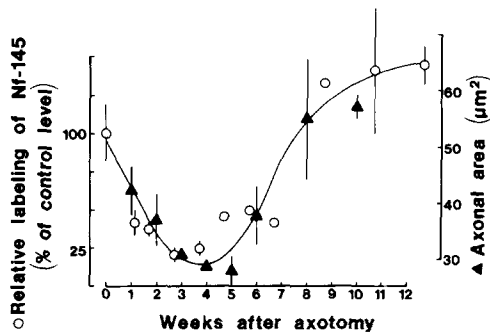


FIGURE 9 Temporal relationship between alterations in axonal caliber and changes in the amount of neurofilament protein transported into the proximal stumps of transected motor fibers. The mean ratios of 145-kD neurofilament protein to tubulin radioactivity in axotomized nerves (○) are normalized in relation to control values and plotted as a function of the time (after axotomy) at which these ratios were measured (i.e., the time at which these animals were killed). The mean cross-sectional areas of the largest 25% of axons in the proximal region of the L5 ventral root were examined at weekly intervals after axotomy and plotted as a function of the time at which animals were killed (Δ). Standard errors of the means are indicated by vertical bars.

are the major cytoskeletal elements in small-caliber (unmyelinated) axons, which contain relatively few neurofilaments (1, 29). In axons of increasing caliber, the number of neuro-

filaments rises faster than do the number of microtubules; consequently, in large-caliber axons neurofilaments outnumber microtubules by more than 10:1 (1, 5). The relative importance of neurofilaments in regulating caliber appears to be greatest in large-caliber axons, which contain the largest number of neurofilaments. In the proximal stumps of transected motor fibers, the greatest reductions in caliber (and neurofilament content) occur in large-caliber axons (i.e., with diameters of 7–9 μm); diameter changes relatively little in small-caliber motor fibers (i.e., with diameters of 3–4 μm), which contain relatively few neurofilaments (8).

Our findings indicate that the axonal transport of neurofilaments plays an important role in the control of axonal caliber in transected motor fibers. Previous studies have shown that reductions in the calibers of these fibers start proximally and proceed anterograde along the proximal stump at a rate equal to the velocity of neurofilament transport (8). In the present study we demonstrated that reductions in caliber coincided with decreased delivery of neurofilament proteins to the proximal stump, while resumption of the radial growth of axons coincided with a return of delivery to pre-axotomy levels.

Our interpretation of these observations assumes that reduction in caliber does not result from the increased turnover of neurofilaments in the proximal stumps of transected motor fibers. This assumption is supported by morphometric studies

suggesting that axotomy does not produce a generalized reduction in neurofilament numbers (i.e., increased degradation of neurofilaments) all along the axon. Axonal caliber and neurofilament content are unchanged until the neurofilament-depleted slow component reaches any given segment of the proximal stump (8). Thus, we conclude that primary reduction in caliber does not lead to a later secondary decrease in neurofilament synthesis and/or transport, but rather that reduction in caliber is related directly to a decrease in the amount of neurofilament protein transported in the proximal stumps.

Moving vs Stationary Neurofilaments in the Cytoskeleton

Our results suggest that the majority of the neurofilaments in lumbar motor axons continuously move in the anterograde direction in the slow component of axonal transport. This conclusion is consistent with the hypothesis of Weiss and Hiscoe (30) that axoplasm continuously moves at a slow rate in the anterograde direction. It is also consistent with the results of radioisotope studies in which pulse-labeled neurofilament proteins form a discrete peak that moves along the nerve as part of the slow component (18, 31, 32), presumably in the form of assembled neurofilaments (24).

Although our observations are most consistent with a model in which the majority of neurofilaments in these motor fibers are continuously moving, they do not exclude the possibility that these fibers also contain a small proportion of stationary neurofilaments. Recently, Nixon et al. (33) found that pulse-labeled neurofilament proteins are locally retained along axons in mouse optic nerve after passage of the slow component wave. They proposed that the accumulation of neurofilaments derived from the transported population could in time result in a substantial proportion of stationary neurofilaments in these axons. Perhaps the relative proportion of stationary neurofilaments is greater in small-caliber axons such as those in mouse optic nerve, which have a mean diameter of 0.5–1.5 μm (Nixon, R. A., personal communication); large-caliber motor axons in rat L5 ventral root are considerably larger (mean diameter, 7 μm) (8).

Velocity of Neurofilament Transport in Regenerating Motor Fibers

The velocity of neurofilament transport was greater in regenerating motor fibers than in controls. At 5 d after labeling, neurofilament proteins extended more distally in the proximal stumps of transected nerves than in control nerves. This difference in velocity after axotomy was not identified previously because transport was examined at a later time after labeling (i.e., 20 d) (7). The greater velocity of neurofilament transport in regenerating fibers than in controls could represent a true increase in the velocities of these proteins in regenerating fibers. Alternatively, it could reflect an absence of the progressive decline in neurofilament velocity with increasing distance along the nerve recently observed in control motor fibers (17).

Regulation of Neurofilament Transport

Our findings are consistent with the hypothesis that neuron–target cell interactions may help to regulate axonal caliber by influencing the level of neurofilament transport (and pre-

sumably synthesis). Decrease in the amount of neurofilament protein transported in axotomized nerve fibers correlates temporally with loss of the functional connection between lumbar motor neurons and muscle. Similarly, return of transport to pre-axotomy levels correlates with reinnervation and recovery of motor function. These observations suggest that interactions between neurons and their targets may play an important role in regulating axonal caliber through their influence on the levels of neurofilament synthesis and transport.

The authors thank Philip Landes, Kenneth Fahnestock and Adelaine Stocks for their excellent technical assistance. We also thank Drs. Nancy and Ludwig Sternberger for providing neurofilament antibodies.

This work was supported by grants from the U.S. Public Health Service (EY 03791, NS 10580, NS 15721, NS 14784, and NS 20164) and the Vanderklute and Dunning funds. Dr. Hoffman is a John A. and George L. Hartford Foundation Fellow, an Alfred P. Sloan Foundation Fellow, and the recipient of a Research Career Development Award from the National Institutes of Health (NS 00896). Dr. Griffin is the recipient of a Research Career Development Award from the National Institutes of Health (NS 00450).

Received for publication 20 December 1984, and in revised form 25 February 1985.

REFERENCES

- Berthold, C. H. 1978. Morphology of normal peripheral axons. In *Physiology and Pathobiology of Axons*. S. G. Waxman, editor. Raven Press, New York. 3–63.
- Friede, R. L., and A. J. Martinez. 1970. Analysis of the process of sheath expansion in swollen nerve fibers. *Brain Res.* 19:165–182.
- Stuart, D. G., and R. M. Enoka. 1983. Motoneurons, motor units, and the size principle. In *The Clinical Neurosciences*. Volume 5. Neurobiology. R. N. Rosenberg, editor. Churchill Livingstone, Inc., New York. 471–517.
- Hursh, J. B. 1939. Conduction velocity and diameter of nerve fibers. *Amer. J. Physiol.* 127:131–139.
- Friede, R. L., and T. Samorajski. 1970. Axon caliber relates to neurofilaments and microtubules in sciatic nerve fibers of rats and mice. *Anat. Rec.* 167:379–387.
- Weiss, P. A., and R. Mayr. 1971. Organelles in neuroplasmic ("axonal") flow: neurofilaments. *Proc. Natl. Acad. Sci. USA.* 68:846–850.
- Hoffman, P. N., and R. J. Lasek. 1980. Axonal transport of the cytoskeleton in regenerating motor fibers: constancy and change. *Brain Res.* 202:317–333.
- Hoffman, P. N., J. W. Griffin, and D. L. Price. 1984. Control of axonal caliber by neurofilament transport. *J. Cell Biol.* 99:705–714.
- Greenman, M. J. 1913. Studies on the regeneration of the peroneal nerve of the albino rat: number and sectional areas of fibers: area relation of axis to sheath. *J. Comp. Neurol.* 23:479–513.
- Gutmann, E., and F. K. Sanders. 1943. Recovery of fiber numbers and diameters in the regeneration of peripheral nerves. *J. Physiol. (Lond.)* 101:489–518.
- Cragg, B. G., and P. K. Thomas. 1961. Changes in conduction velocity and fiber size proximal to peripheral nerve lesions. *J. Physiol. (Lond.)* 157:315–327.
- Aitkin, J. T., and P. K. Thomas. 1962. Retrograde changes in fiber size following nerve section. *J. Anat.* 96:121–129.
- Kreutzberg, G. W., and P. Schubert. 1971. Volume changes in the axon during regeneration. *Acta Neuropathol. (Berl.)* 17:220–226.
- Kuno, M., Y. Miyata, and E. J. Munoz-Martinez. 1974. Differential reactions of fast and slow alpha-motoneurons to axotomy. *J. Physiol. (Lond.)* 240:725–739.
- Carlson, J., A. C. Lais, and P. J. Dyck. 1979. Axonal atrophy from permanent peripheral axotomy in adult cat. *J. Neuropathol. Exp. Neurol.* 38:579–585.
- Dyck, P. J., H. Nukada, A. C. Lais, and J. L. Karnes. 1984. Permanent axotomy: a model of chronic neuronal degeneration preceded by axonal atrophy, myelin remodeling, and degeneration. In *Peripheral Neuropathy*. P. J. Dyck, P. K. Thomas, E. H. Lambert, and R. Bunge, editors. W. B. Saunders Co., Philadelphia. 666–690.
- Hoffman, P. N., J. W. Griffin, B. G. Gold, and D. L. Price. 1985. Slowing of neurofilament transport and the radial growth of developing nerve fibers. *J. Neurosci.* In press.
- Hoffman, P. N., and R. J. Lasek. 1975. The slow component of axonal transport: identification of the major structural polypeptides of the axon and their generality among mammalian neurons. *J. Cell Biol.* 66:351–366.
- Griffin, J. W., D. C. Anthony, K. E. Fahnestock, P. N. Hoffman, and D. G. Graham. 1984. 3, 4-Dimethyl-2, 5-hexanedione impairs the axonal transport of neurofilament proteins. *J. Neurosci.* 4:1516–1526.
- Goldstein, M. E., L. A. Sternberger, and N. H. Sternberger. 1983. Microheterogeneity ("neurotypy") of neurofilament proteins. *Proc. Natl. Acad. Sci. USA.* 80:3101–3105.
- Sternberger, L. A., and N. H. Sternberger. 1983. Monoclonal antibodies distinguish phosphorylated and nonphosphorylated forms of neurofilaments *in situ*. *Proc. Natl. Acad. Sci. USA.* 80:6126–6130.
- Willard, M. 1983. Neurofilaments and axonal transport. In *Neurofilaments*. C. A. Marotta, editor. University of Minnesota Press, Minneapolis. 86–116.
- Sinicropi, D. V., and D. L. McIlwain. 1983. Changes in the amounts of cytoskeletal proteins within the perikarya and axons of regenerating frog motoneurons. *J. Cell Biol.* 96:240–247.
- Morris, J. R., and R. J. Lasek. 1982. Stable polymers of the axonal cytoskeleton: the axoplasmic ghost. *J. Cell Biol.* 92:192–198.

25. Hoffman, P. N., J. W. Griffin, and D. L. Price. 1984. Neurofilament transport in axonal regeneration: implications for the control of axonal caliber. *In* *Advances in Neurochemistry, Axonal Transport in Growth and Regeneration*. J. Elam and P. Cancalon, editors. Plenum Publishing Corp., New York. 243-260.
26. Zenker, W., R. Mayr, and H. Gruber. 1972. Axoplasmic organelles: quantitative differences between ventral and dorsal root fibers of the rat. *Experientia*. 29:77-78.
27. Sasaki-Sherrington, S. E., J. R. Jacobs, and J. K. Stevens. 1984. Intracellular control of axial shape in non-uniform neurites: a serial electron microscopic analysis of organelles and microtubules in AI and AII retinal amacrine cells. *J. Cell Biol.* 98:1279-1290.
28. Nadelhaft, I. 1974. Microtubule densities and total numbers in selected axons of the crayfish abdominal nerve cord. *J. Neurocytol.* 3:73-86.
29. Peters, A., and J. E. Vaughn. 1967. Microtubules and filaments in the axons and astrocytes of early postnatal optic nerves. *J. Cell Biol.* 32:113-119.
30. Weiss, P. A., and H. B. Hiscoe. 1948. Experiments on the mechanism of nerve growth. *J. Exp. Zool.* 107:315-396.
31. Mori, H., Y. Komiya, and M. Kurokawa. 1979. Slowly migrating axonal polypeptides. Inequalities in their rate and amount of transport between two branches of bifurcating axons. *J. Cell Biol.* 82:174-184.
32. Black, M. M., and R. J. Lasek. 1980. Slow components of axonal transport: two cytoskeletal networks. *J. Cell Biol.* 86:616-623.
33. Nixon, R. A., K. A. Bishop, and W. H. Fisher. 1983. Retention of axonally transported proteins along mouse retinal ganglion cell axons. *Soc. Neurosci. Abstr.* 9:149.
34. Chiu, F.-C., and W. T. Norton. 1982. Bulk preparation of CNS cytoskeleton and separation of individual neurofilament proteins by gel filtration: dye-binding characteristics and amino acid compositions. *J. Neurochem.* 39:1252-1260.
35. Shecket, G., and R. J. Lasek. 1980. Preparation of neurofilament protein from guinea pig peripheral nerve and spinal cord. *J. Neurochem.* 35:1335-1344.