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

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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 intrasural 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. 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 x 10^9 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 spectropy.

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 ul of a solution containing 8 M urea, 5% 2-mercaptoethanol, and 30 mM sodium phosphate, pH 6.5. Homogenates were incubated in a boiling water bath for 4 min. Aliquots (40 ul) of the original homogenate and of serial dilutions of the homogenate (i.e., 1/2, 1/4, 1/16, and 1/8) were dotted onto nitrocellulose membranes using a Hybrid-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 Des. 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 AB, Bromma, Sweden. Bands 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-μm 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).
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 (O) 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.

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.

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 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
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 \( \mu \text{m}^2/\text{wk} \) 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 \( \mu \text{m}^2/\text{wk} \) 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.

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 μm², 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 μm) (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...
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 \( \mu m \)); diameter changes relatively little in small-caliber motor fibers (i.e., with diameters of 3–4 \( \mu m \)), 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) along all 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 presumably 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.

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