Axonal Transport of Class II and III β-tubulin: Evidence That the Slow Component Wave Represents the Movement of Only a Small Fraction of the Tubulin in Mature Motor Axons

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Abstract. Pulse-labeling studies demonstrate that tubulin synthesized in the neuron cell body (soma) moves somatofugally within the axon (at a rate of several millimeters per day) as a well-defined wave corresponding to the slow component of axonal transport. A major goal of the present study was to determine what proportion of the tubulin in mature motor axons is transported in this wave. Lumbar motor neurons in 9-wk-old rats were labeled by injecting [^{35}S]methionine into the spinal cord 2 wk after motor axons were injured (axotomized) by crushing the sciatic nerve. Immunoprecipitation with mAbs which recognize either class II or III β-tubulin were used to analyze the distributions of radioactivity in these isotypes in intact and axotomized motor fibers 5 d after labeling. We found that both isotypes were associated with the slow component wave, and that the leading edge of this wave was enriched in the class III isotype. Axotomy resulted in significant increases in the labeling and transport rates of both isotypes. Immunohistochemical examination of peripheral nerve fibers demonstrated that nearly all of the class II and III β-tubulin in nerve fibers is located within axons. Although the amounts of radioactivity per millimeter of nerve in class II and III β-tubulin were significantly greater in axotomized than in control nerves (with increases of +160% and +58%, respectively), immunoassay revealed no differences in the amounts of these isotypes in axotomized and control motor fibers. We consider several explanations for this paradox; these include the possibility that the total tubulin content is relatively insensitive to changes in the amount of tubulin transported in the slow component wave because this wave represents the movement of only a small fraction of the tubulin in these motor fibers.

MICROTUBULES play important roles in the growth and maintenance of the neuronal axon (Mitchison and Kirschner, 1988). Tubulin, the principal protein subunit of microtubules, is synthesized in the neuron cell body (soma). Newly synthesized tubulin labeled by injecting radioactive amino acids in the immediate vicinity of neuron cell bodies moves somatofugally within axons at a rate of several millimeters per day in a well-defined wave corresponding to the slow component of axonal transport (Hoffman and Lasek, 1975).

A major goal of the present study was to determine what proportion of the tubulin in mature motor fibers is transported in the slow component wave. The observation that radioactive tubulin remains along the motor axons of young (3-wk-old) rats after passage of the slow component wave (see Fig. 1 of Hoffman et al., 1985*a*) indicates that these motor fibers contain tubulin delivered by slow axonal transport which either becomes stationary or continues to move at slower rates (Watson et al., 1990).

Axonal injury (axotomy) results in an increase in the abundance of tubulin mRNAs in peripheral motor neurons (Muma et al., 1990; Tetzlaff et al., 1991). This is associated with a rise in the labeling of transported tubulin and a broadening of the pulse-labeled tubulin wave, which extends farther distally in the proximal stumps of axotomized than control motor fibers (Hoffman and Lasek, 1980; Hoffman et al., 1985*b*; Tashiro and Komiya, 1991). In spite of this apparent increase in the amount of transported tubulin, morphological analyses reveal no increase (compared with controls) in the number of microtubules in the proximal stumps of axotomized motor fibers (Hoffman et al., 1984). This suggests either that the axonal tubulin content is unchanged after axotomy or that axotomized motor fibers contain increased amounts of unassembled tubulin.

The tubulin molecule is a dimer comprised of a single α and β polypeptide. α- and β-tubulin are each encoded by multigene families (Cleveland, 1987). At least six isotypes of β-tubulin can be distinguished in vertebrates on the basis

of their amino acid sequences (Sullivan and Cleveland, 1986; Lopata and Cleveland, 1987). Four of these isotypes are present in axons (Joshi and Cleveland, 1989); two (classes II and III) appear to play a special role in axonal growth. The expression of class II (Hoffman and Cleveland, 1988) and class III β-tubulin (P. N. Hoffman and M. A. Lopata, unpublished observations) selectively increases in peripheral neurons after axotomy, and both isotypes accumulate in cultured neurons during neurite outgrowth (Joshi and Cleveland, 1989).

In the present study, immunoprecipitation with mAbs which specifically recognize the carboxy-terminal sequences of either class II (Banerjee et al., 1988) or class III β-tubulin (Banerjee et al., 1990) was used to characterize the axonal transport of these isotypes when labeled in axotomized and control motor neurons of rat. Immunohistochemistry confirmed that nearly all of the class II and III β-tubulin in peripheral nerve fibers is located within axons. This allowed us to use immunoassay to compare the total amounts of class II and III β-tubulin in axotomized and control motor fibers. Although we found that the amounts of radioactivity per millimeter of nerve in class II and III β-tubulin were significantly greater in axotomized than in control motor fibers, immunoassay revealed no significant differences in the levels of these isotypes in axotomized and control motor fibers. These findings suggest that the slow component wave represents the movement of only a small fraction of the tubulin in mature motor fibers.

Materials and Methods

Sciatic Nerve Crush

Male Sprague-Dawley rats were used in these studies. All surgical procedures were carried out using chloral hydrate anesthesia (400 mg/kg, i.p.). The left sciatic nerves of 7-wk-old animals were crushed twice for 30 s at the junction of the fourth and fifth lumbar (L4 and L5) spinal roots (~50 mm from the spinal cord) using No. 7 Dumont forceps (Roboz Surgical Instruments, Inc., Washington, D.C.). The contralateral nerve was left undisturbed as a control. Motor axons of the sciatic nerve arise from neurons in the lumbar spinal cord. After leaving the spinal cord, these motor axons are distributed in the L4 and L5 ventral roots, where they are segregated from sensory axons (which are located in the dorsal roots). At the level of the dorsal root ganglia (~30 mm from the spinal cord), motor and sensory axons (i.e., from the ventral and dorsal roots, respectively) intermix to form the spinal roots which subsequently join to form the main trunk of the sciatic nerve.

Labeling Motor Neurons

The lumbar spinal cord was exposed by laminectomy, and motor neurons were labeled by the intraspinal injection of [^{35}S]methionine (New England Nuclear, Boston, MA) (>800 Ci/mmole) according to published methods (Hoffman and Lasek, 1975). This isotope was dried in a vacuum centrifuge and stored dry at −80°C until the time of injection. 1 μl of isotope (diluted to a final concentration of 100 μCi/μl with distilled water) was injected at each site over a period of 6–10 min. Axotomized motor neurons were labeled 14 d after axotomy. In most cases, intact motor neurons were labeled in previously unoperated, age-matched (9-wk-old) control animals. In these animals, four or five injections were made on one side (left) of the spinal cord. In some experiments, the labeling of transported proteins was compared in axotomized motor fibers and contralateral control fibers. In these animals, isotope was injected symmetrically at three sites on each side of the spinal cord (i.e., a total of six injections). In all cases, animals were killed 5 d after injection using an overdose of anesthesia. The labeled sciatic nerve and roots were removed and stored at −80°C.

Western Blots

Proteins were analyzed on SDS-polyacrylamide gels (8.5% acrylamide) according to the method of Laemmli (1970) and transferred to nitrocellulose membranes (Schleicher and Schuell, Inc., Keene, NH) at 20 V for 14 h. All incubations were carried out at room temperature. The blot was first incubated for 4 h in a buffer containing 4% BSA, 0.2% Triton X-100, 150 mM NaCl, 1 mM EGTA, and 10 mM phosphate buffer, pH 7.5, followed by incubation overnight in the same buffer containing mAbs diluted to appropriate concentrations (1:1,000 for the clone DM1B β-tubulin antibody [Amersham Corp., Arlington Heights, IL] 1:1,500 for the clone JDR-3B8 class II antibody [Banerjee et al., 1988] and the clone SDL3D10 class III antibody [Banerjee et al., 1990]). mAbs not specifically bound were removed from the blot by five 3-min-long washes in Solution A (0.5% Triton X-100, 0.1% SDS, 100 mM NaCl, 2 mM EDTA, and 50 mM triethanolamine, pH 7.4). The blot was next incubated for 1 h in ^{125}I-labeled goat anti-mouse secondary antibody (Amersham Corp.) diluted 1:1,000; nonspecifically bound antibody was removed by five 3-min-long washes in Solution A. The blot was then dried and exposed to Kodak X-OMAT AR (Eastman Kodak Co., Rochester, NY) film for 4–16 h using a DuPont Lightening Plus Intensifying Screen (DuPont Co., Wilmington, DE).

Synthetic Peptides

The β-tubulin isotypes are distinguished principally by sequences within the 15 carboxy-terminal amino acid residues. The synthesis and characterization of synthetic peptides corresponding to these variable domains has been described previously (Lopata and Cleveland, 1987). Briefly, peptides were chemically synthesized using a peptide synthesizer. After purification by high-performance liquid chromatography, sequences of each peptide were verified by analysis of amino acid composition. The synthetic peptides for the class II and III isotypes had the following amino acid sequences (using the one letter amino acid code): EEEEGEDEA and GEMYEDDEEESESQGPK, respectively (Lopata and Cleveland, 1987).

Cloned Fusion Proteins Containing the Carboxy-terminal Sequences of the β-tubulin Isotypes

For each of the β-tubulin isotypes, a plasmid was constructed which carried a chimeric gene that directed the synthesis of a fusion protein in bacteria. The construction of expression plasmids and the induction of expression of these plasmids has been described previously (Lopata and Cleveland, 1987). Briefly, each fusion protein contained the amino-terminal 32 kD of the bacterial protein trpE linked to amino acid residues from β-tubulin starting at codon 345 through the carboxy terminus. These plasmids were transformed into *Escherichia coli*, and expression was controlled through the trpE promoter.

Immunohistochemical Localization of Class II and III β-tubulin in Peripheral Nerve Fibers

Animals were anesthetized with chloral hydrate before intracardiac perfusion with 4% paraformaldehyde and 2.5% glutaraldehyde. Segments of the sciatic nerve were removed, embedded in Epon, and cut into 1-μm-thick sections. Epon was removed from the sections using sodium ethoxide (Trapp et al., 1981). Sections were stained by the avidin-biotin-peroxidase complex technique (Hsu et al., 1981) using horse anti-mouse antibody and mouse mAbs specific for either class II or III β-tubulin at dilutions of 1:1,000.

The Axonal Transport of Tubulin Isotypes along Motor Fibers

Sciatic nerves and motor roots containing ^{35}S-labeled proteins were cut into consecutive 3-mm-long segments and homogenized in 50 μl of immunoprecipitation buffer (0.5% SDS, 0.1% deoxycholate, 1% NP-40, 400 mM NaCl, and 25 mM Tris, pH 7.4) using a glass-on-glass microhomogenizer (Micrometrics Instruments Corp., Tampa, FL). The homogenates were transferred to Eppendorf tubes and centrifuged at 14,000 rpm for 5 min. Western blot analyses demonstrated that essentially all of the tubulin in the nerve homogenates was recovered in the resulting supernatant fractions. The insoluble pellets, which were largely devoid of tubulin, were solubilized in Laemmli gel sample buffer containing 6 M urea before analysis by SDS-PAGE. Aliquots of the supernatant were used for analysis of labeled proteins by SDS-PAGE (5% of total), liquid scintillation spectroscopy (3.5%), and immunoprecipitation (the remaining 91.5%).

Immunoprecipitation of Labeled Tubulins

For each immunoprecipitation reaction, 15 μl of nerve extract in immunoprecipitation buffer was added to 35 μl of PBS containing an appropriate amount of mAb (2 μl for DM1B, 3 μl for class II and 4 μl for class III) and allowed to incubate for 16 h at room temperature. Tachisorb (Calbiochem Corp., LaJolla, CA) was washed through centrifugation and resuspended in PBS at a concentration that assured that the 100-μl aliquot added to the reaction mixture contained the appropriate amount (200 μl of prewashed Tachisorb for the DM1B and class III antibodies and 400 μl for the class II antibody). After incubation for 1 h at room temperature, the reaction mixture was layered over 150 μl of 1 M sucrose in PBS and spun in an Eppendorf centrifuge at 14,000 rpm for 5 min. The Tachisorb/antibody/tubulin-containing pellets were resuspended in Laemmli gel buffer (solubilizing antibody and tubulin), boiled for 2 min, and analyzed by SDS-PAGE on 8.5% gels. The gels were fluorographed (Bonner and Laskey, 1974) and the immunoprecipitated tubulin gel bands were detected using Kodak X-OMAT AR film after exposure for 1–4 d. The tubulin bands were removed from the gels, dissolved in 30% H_2O_2 at 60°C for 48 h, and levels of radioactivity determined using liquid scintillation spectroscopy. To insure that only adequately labeled nerves were included in our analyses, we excluded any nerves in which the total level of radioactivity in immunoprecipitated class II β-tubulin was at least one standard deviation less than the population mean.

For each nerve included in our analyses, the levels of radioactivity in tubulin gel bands were normalized in relation to the total for that isotype (or total β-tubulin in the case of the DM1B antibody). The means of the normalized values were plotted as a function of distance along the nerve; they were also plotted cumulatively as a function of distance along the nerve (Hoffman et al., 1983). The locations of the 50th and 75th percentiles of radioactivity at 5 d after labeling were determined for each nerve, and mean values were calculated. The location of the 50th percentile of radioactivity was used to calculate the median transport rate (mm/d), which was calculated by dividing the distance (mm) by the postlabeling interval (5 d). The location of the 75th percentile of radioactivity was used to evaluate the behavior of the leading edge of the tubulin wave.

Measuring the Labeling of Tubulin and Neurofilament Proteins in Axotomized and Control Motor Fibers

Axotomized and contralateral control motor neurons were labeled 14 d after crushing one sciatic nerve. 5 d after labeling, animals were killed with an overdose of anesthesia and both the axotomized and contralateral control sciatic nerves and roots were removed. At this time interval, essentially all of the radioactive tubulin and neurofilament (NF)[1] protein in both axotomized and control motor fibers was located in a region extending 0–25 mm from the spinal cord (see Figs. 5 and 6). Segments extending 0–25 mm along the L4 and L5 ventral roots of each nerve were combined and homogenized in 200 μl of a solution containing 150 mM NaCl, 25 mM Tris, pH 7.4, using a glass-on-glass microhomogenizer. A 30-μl aliquot of this homogenate was used to prepare labeled cytoskeletal proteins. This aliquot was placed in a separate homogenizer, brought to final volume of 60 μl in a solution containing 25 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.9 M sucrose, 1 mM DTT, 1 mM PMSF, and 1 mM EGTA, rehomogenized, transferred to an Eppendorf tube, and centrifuged at 14,000 rpm for 30 min at 4°C. Equal proportions of the resulting pellet and supernatant fractions were analyzed using SDS-PAGE and gel fluorography. Essentially all of the labeled NF proteins were located in the pellet fractions. The gel bands corresponding to NF-L, NF-M, and NF-H were removed from the gels, dissolved in 30% H_2O_2, and the levels of radioactivity measured using liquid scintillation spectroscopy.

The remaining homogenate (170 μl) was brought to a final volume of 340 μl in immunoprecipitation buffer (i.e., 0.5% SDS, 0.1% deoxycholate, 1% NP-40, 400 mM NaCl, and 25 mM Tris, pH 7.4), and rehomogenized. The homogenate was then transferred to an Eppendorf tube and centrifuged at 14,000 rpm for 5 min. 15-μl aliquots of the resulting supernatant fractions, containing labeled tubulin, were analyzed by immunoprecipitation and SDS-PAGE. Tubulin bands were removed from the gels, dissolved in 30% H_2O_2, and levels of radioactivity measured using liquid scintillation spectroscopy.

1. *Abbreviation used in this paper*: NF, neurofilament.

Determining the Amounts of Radioactivity per Millimeter of Nerve in Class II and III β-tubulin

Since axotomy-induced changes in neuronal mRNA levels correlate with alterations in the incorporation of labeled precursors into newly synthesized cytoskeletal proteins (Hoffman et al., 1987; Greenberg and Lasek, 1988; Wong and Oblinger, 1987), it is likely that the amounts of class II and III β-tubulin undergoing transport are proportional to the levels of transported radioactivity. The quantity Q, which is an index of the amount of radioactivity per unit length of nerve (cpm/mm), can be obtained by dividing the total amount of radioactivity in the nerve (see Table II) by the distance that the 50th percentile of radioactivity has migrated during the 5-d postlabeling interval (see Table I). In this way, the amount of transported radioactivity per unit length of nerve can be compared in axotomized and control motor fibers for each isotype.

Immunoassay of Tubulin

At either 5 or 19 d after crushing one sciatic nerve, animals were killed with an overdose of anesthesia and ventral roots were removed bilaterally and stored at −80°C. The 19-d interval corresponds to the time at which transport was analyzed in axotomized motor fibers (i.e., proteins were labeled 14 d after axotomy, and their distributions analyzed 5 d later). 4-mm-long segments harvested from both proximal and distal regions of L5 ventral roots from axotomized and contralateral control nerves were homogenized in 200 μl of 8 M urea, 5% β-mercaptoethanol, and 20 mM phosphate buffer, pH 7.4.

Immunoassay of the content of total β-tubulin was performed using the DM1B antibody as previously described (Watson et al., 1989, 1991). In addition, the mAbs specific for class II and III β-tubulin were used at 1:2,000 dilution in a parallel immunoassay. Triton X-100 insoluble cytoskeletal proteins prepared by axonal flotation of roots in the cauda equina were used as NF standards. Taxol-stabilized, salt-washed microtubules polymerized from rat brain proteins (Vallee, 1982) were used as tubulin standards.

In brief, homogenates were diluted 200-fold in 0.8 M urea, 0.5% β-mercaptoethanol and allowed to bind to polylysine-coated microliter wells. Subsequent blocking with normal serum, binding to primary mAb and detection with biotinylated anti-mouse antibody and avidin-biotin peroxidase complexes were performed as previously described. The results are expressed in OD units. For total β-tubulin, 1 OD = 1 μg of protein. For the class II and III isotypes, 1 OD unit corresponds to the amount of that isotype in 1 μg of total β-tubulin in the ventral roots.

Results

The aims of the present study were to compare in axotomized and control motor fibers: (*a*) the levels of radioactivity in transported class II and III β-tubulin; (*b*) the transport rates of these proteins; and (*c*) the total amounts of these proteins in motor fibers. It was first necessary to confirm that the antibodies used in this study specifically recognize class II and III β-tubulin, respectively. This was accomplished by demonstrating that each antibody was able to recognize in a specific manner the bacterial fusion protein containing the carboxy-terminal sequence specific for that isotype (Lopata and Cleveland, 1987) (Fig. 1). This analysis also demonstrated that the DM1B antibody recognized the fusion proteins for all four β-tubulin isotypes examined, but not the 32-kD bacterial fusion protein vector (Fig. 1). This is consistent with the demonstration that the DM1B antibody recognizes an epitope which is common to all β-tubulin isotypes (Lopata and Cleveland, 1987; de la Vina et al., 1988).

Immunohistochemical analyses of peripheral nerve fibers demonstrated that essentially all of the class II β-tubulin in peripheral nerve fibers is localized within axons (Fig. 2). This is consistent with the results of in situ hybridization studies demonstrating that mRNAs encoding this isotype are detectable in neurons, but not in Schwann cells or other cell types in peripheral nerve fibers (Hoffman and Cleveland, 1988). In addition, nearly all of the class III β-tubulin in pe-

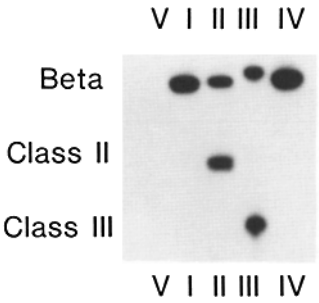

Figure 1. Western blot analysis using bacterial lysates containing 100 ng of fusion proteins for the class I, II, III, and IV isotypes of β-tubulin (including the 32-kD fusion protein vector). The class II and III antibodies recognized their respective isotypes. The DM1B antibody (*Beta*) recognized all four isotypes. The production of these fusion proteins has been described previously (Lopata and Cleveland, 1987). *I*, class I fusion protein; *II*, class II fusion protein; *III*, class III fusion protein; *IV*, class IV fusion protein; and *V*, 32-kD fusion protein vector.

ripheral nerve fibers was located within axons. Only minor levels of immunoreactivity for the class III isotype were noted outside of axons (i.e., in Schwann cell cytoplasm) (Fig. 2). This predominantly neuronal localization of the class III isotype has been noted previously (Lee et al., 1990). The presence of essentially all of the class II and most of the class III isotype in axons allowed us to use immunoassay to compare the total amounts of these isotypes in axotomized and control motor fibers.

We determined whether these antibodies could be used to immunoprecipitate class II and III β-tubulin in a specific manner. This was accomplished by demonstrating that synthetic peptides bearing the sequences of the carboxy-terminal amino acid residues of either class II or III β-tubulin (Lopata and Cleveland, 1987) were capable of selectively blocking the immunoprecipitation of labeled tubulin by their respective antibodies (Fig. 3). (For the amino acid sequences of these peptides see Materials and Methods.) Immunoprecipitation of labeled bands with the molecular weight of tubulin was blocked using the appropriate peptides. For example, immunoprecipitation of tubulin with the antibody directed against the class II isotype was specifically blocked by the class II–specific peptide. Note that a band with a molecular weight higher than that of tubulin was also immunoprecipitated by the class II and III antibodies, and that immunoprecipitation of this band was not blocked by either peptide (Fig. 3). Since levels of radioactivity were measured

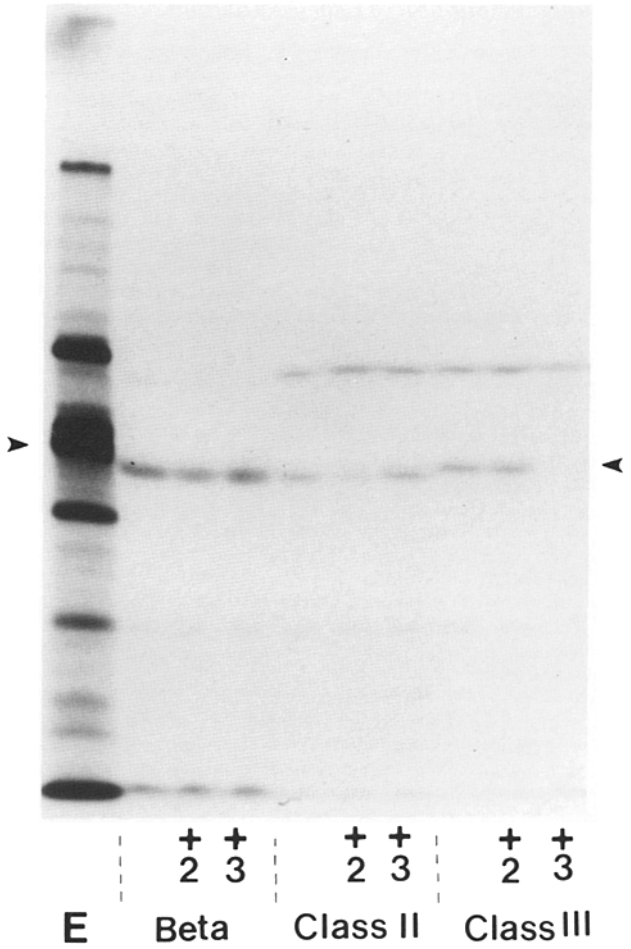

Figure 3. Specific blocking of immunoprecipitation by synthetic peptides. Supernatant extracts of nerve homogenates containing labeled proteins (10^4 cpm) were immunoprecipitated either with (+) or without the addition of peptides with sequences specific for the carboxy-terminals of either class II (+2) or III (+3) β-tubulin. The synthesis and characterization of these peptides have been described previously (Lopata and Cleveland, 1987). The position of the tubulin bands is indicated by arrows. *E*, supernatant extract before immunoprecipitation; *Beta*, proteins immunoprecipitated by the DM1B antibody; *Class II*, proteins immunoprecipitated by the class II antibody; and *Class III*, proteins immunoprecipitated by the class III antibody. Peptide concentrations, 0.5 mg/ml.

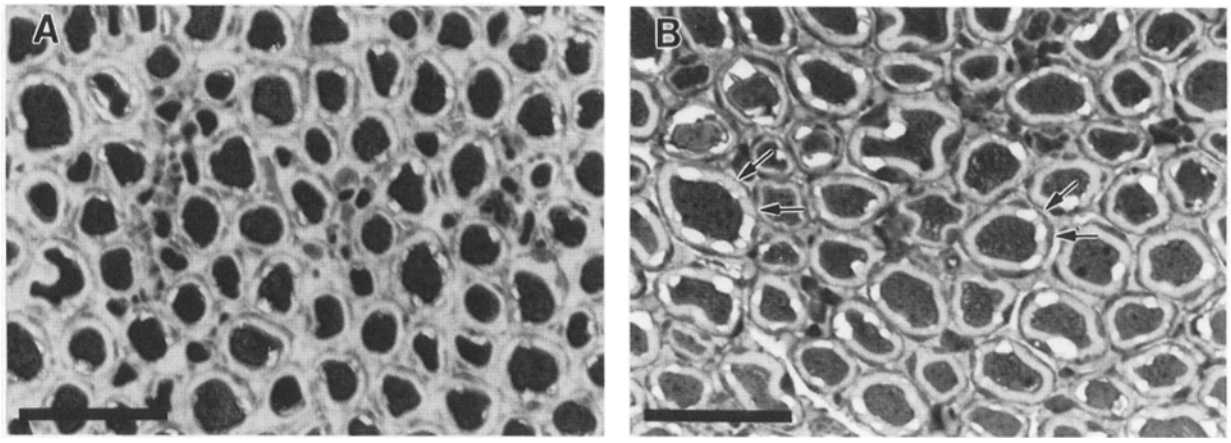

Figure 2. Immunohistochemical localization of class II (*A*) and III (*B*) β-tubulin in sciatic nerve axons. Axons are darkly stained, indicating that they contain abundant avidin–biotin–peroxidase reaction product. The myelin sheaths surrounding these axons are unstained (*arrows*). Schwann cell cytoplasm contains low levels of staining for the class III isotype. Section thickness, 1 μm. Bars, 50 μm.

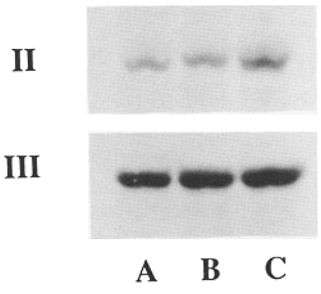

Figure 4. Fluorograms demonstrating that class II and III β-tubulin are immunoprecipitated under conditions of antibody excess. The immunoprecipitation of labeled tubulin was compared in samples containing the same amounts of labeled tubulin, but differing in their total tubulin contents. *A*, the normal concentration of tubulin; *B*, 2× the normal concentration of tubulin; and *C*, 3× the normal concentration of tubulin. Identical amounts of labeled tubulin were immunoprecipitated from each sample by either the class II or III antibodies. *II*, tubulin immunoprecipitated by the class II antibody; *III*, tubulin immunoprecipitated by the class III antibody. Fluorograms were exposed for 7 d.

exclusively in the gel band with a molecular weight corresponding to that of tubulin, this higher molecular weight band did not influence our analyses of tubulin transport.

We next asked whether the immunoprecipitation of class II and III β-tubulin was carried out under conditions of antibody excess. This was investigated by comparing the amounts of labeled tubulin immunoprecipitated from samples containing the same amounts of labeled tubulin, but differing in their total tubulin contents. To do this, homogenates of either labeled or unlabeled nerve segments which were 3× more concentrated than normal (i.e., 9-mm-long nerve segments were homogenized in 50 μl of immunoprecipitation buffer) were mixed to yield the following samples: (*a*) one part labeled homogenate plus two parts immunoprecipitation buffer (i.e., the normal concentration of tubulin); (*b*) one part labeled homogenate plus one part unlabeled homogenate plus one part immunoprecipitation buffer (i.e., 2× the normal concentration of tubulin); and (*c*) one part labeled homogenate plus two parts unlabeled homogenate (i.e., 3× the normal concentration of tubulin). These analyses revealed that identical amounts of labeled tubulin were immunoprecipitated from each sample by either the class II or III antibodies (Fig. 4). This indicates that immunoprecipitation was carried out under conditions of antibody excess. Therefore, essentially all of the class II and III β-tubulin in these samples was immunoprecipitated under the conditions used in this study. Thus, our results demonstrate the following: (*a*) our antibodies specifically recognize class II and III β-tubulin; (*b*) these isotypes can be specifically immunoprecipitated using these antibodies; and (*c*) immunoprecipitation provides a quantitative measure of the amounts of radiolabeled class II and III β-tubulin in the nerve.

We analyzed the axonal transport of pulse-labeled class II and III β-tubulin in intact motor axons of the L4 and L5 ventral roots, which are the principal source of motor fibers to the sciatic nerve. 5 d after motor neurons were labeled by injecting [^{35}S]methionine into the lumbar spinal cord, animals were killed and the labeled proteins in consecutive, 3-mm-long segments of the L4 and L5 ventral roots were analyzed according to the procedure described in Materials and Methods. Using immunoblots, we confirmed that essentially all of the tubulin in nerve segments was solubilized by homogenization in immunoprecipitation buffer (i.e., the supernatant fractions contained essentially all of the tubulin). The labeled tubulin in supernatant fractions was subjected to immunoprecipitation and the resulting pellets, containing immunoprecipitated tubulin, were analyzed using SDS-PAGE and gel fluorography. Fig. 5 illustrates representative fluorograms comparing the distributions of pulse-labeled class II, class III, and total β-tubulin along the ventral roots 5 d after labeling. The labeled tubulin bands were removed from the gels and their levels of radioactivity measured using liquid scintillation spectroscopy. For each isotype, the levels of radioactivity in each nerve segment were normalized in relation to the total in the nerve. The means of these normal-

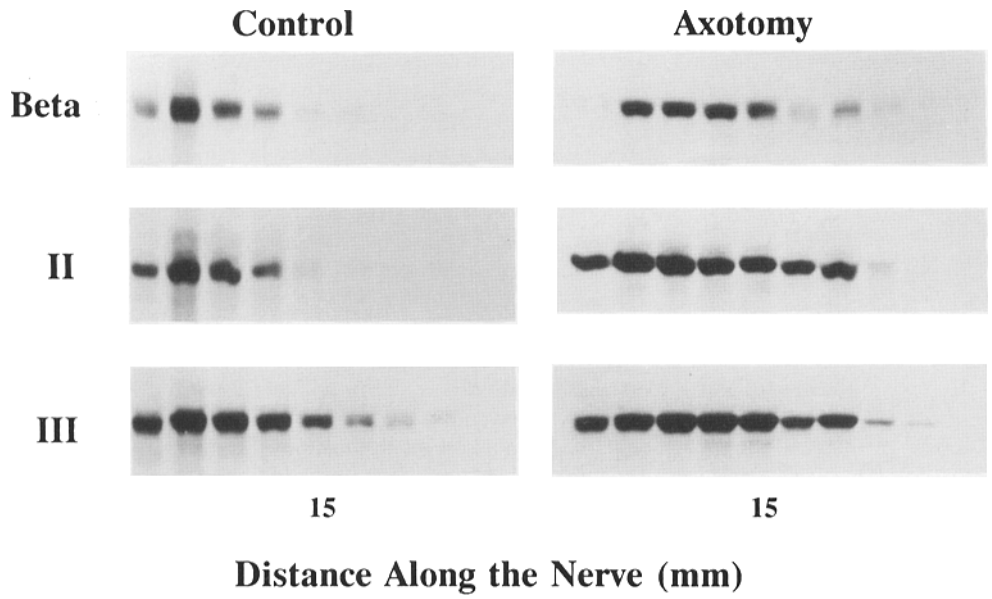

Figure 5. Fluorograms comparing the distributions of labeled class II, class III, and total β-tubulin in the proximal stumps of axotomized and control motor fibers. The labeled tubulin in 3-mm-long segments of the lumbar ventral roots were analyzed by immunoprecipitation and SDS-PAGE 5 d after motor neurons were labeled by injecting [^{35}S]methionine into the spinal cord. Axotomized motor neurons were labeled 14 d after crushing the sciatic nerve. *Beta*, tubulin immunoprecipitated by the DM1B antibody, which recognizes total β-tubulin; *II*, tubulin immunoprecipitated by the class II-specific antibody; and *III*, tubulin immunoprecipitated by the class III-specific antibody. Fluorograms were exposed for 3 d.

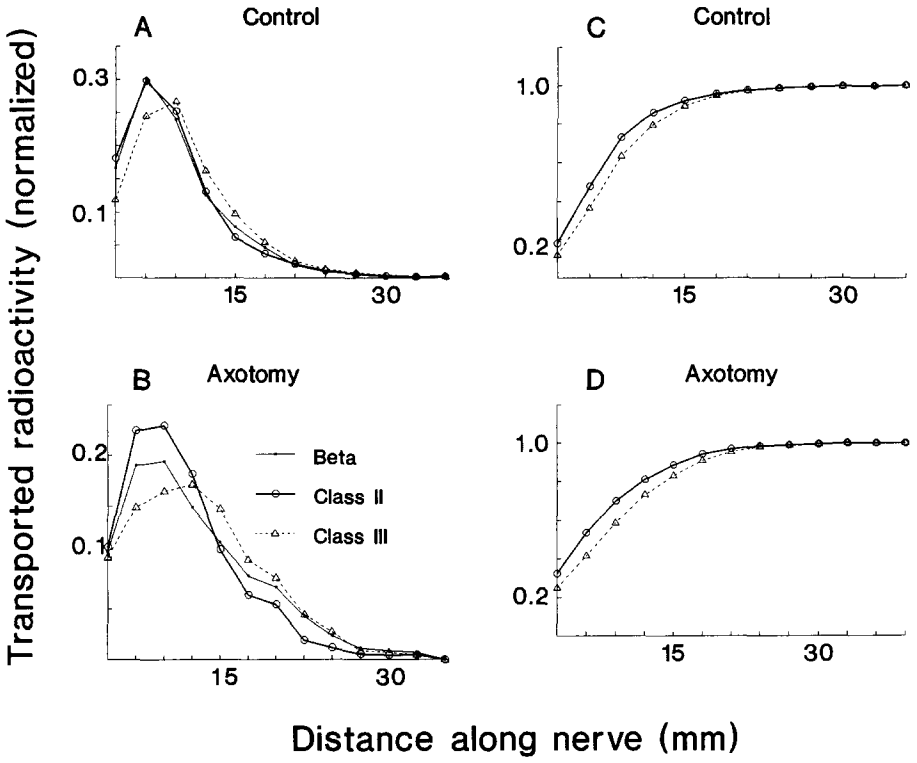

Figure 6. Quantitation of tubulin radioactivity along axotomized and control motor fibers. Gel bands containing labeled tubulins, such as those shown in Fig. 5, were removed from gels, and the levels of radioactivity measured using liquid scintillation spectroscopy. For each isotype, the levels of radioactivity in each nerve segment were normalized in relation to the total in that nerve. The means of these normalized values are plotted as a function of distance along the nerve either for each nerve segment (*A* and *B*) or cumulatively (*C* and *D*). The mean total levels of radioactivity (cpm ± standard errors) immunoprecipitated by the class II, class III, and DM1B (*Beta*) antibodies, respectively, were 4,056 ± 386, 3,682 ± 433, and 6,948 ± 612 for the control nerves (n = 9), and 8,153 ± 811, 8,212 ± 1,078, and 7,410 ± 1,481 for the axotomized nerves (n = 7).

ized values are plotted as a function of distance along the nerve in Fig. 6.

This analysis revealed that pulse-labeled class II and III β-tubulin are transported in the slow component wave (Hoffman and Lasek, 1975) (Fig. 6). Tubulin transport was also examined in axotomized motor neurons labeled 14 d after crushing the sciatic nerve. Comparison of the distributions of labeled tubulin in axotomized and control motor fibers demonstrated that class II, class III, and total β-tubulin extended farther distally in axotomized than in control motor fibers (Figs. 5 and 6). For each isotype, both the 50th and 75th percentiles of radioactivity were located more distally in axotomized than in control motor fibers (Fig. 6, *C* and *D*; Table I). Thus, the median rates at which these isotypes were transported in the slow component wave, which were calculated on the basis of the locations of the 50th percentiles of radioactivity (Table I), were significantly greater in axotomized than in control motor fibers. Since the DM1B antibody recognizes both the class II and III isotypes (Fig. 1), apparent differences in the distributions of labeled tubulins immunoprecipitated by the DM1B antibody and either the class II or III antibodies (Figs. 5 and 6) are likely to reflect differences in the affinities of these antibodies.

*Table I. Location of Tubulin Radioactivity**

	Control	Axotomy	Significance‡
	50th percentile of radioactivity		
Total β	6.43 ± 0.51	9.67 ± 0.85	$P < 0.005$
Class II	6.20 ± 0.47	8.86 ± 0.81	$P < 0.01$
Class III	7.46 ± 0.51	10.79 ± 0.96	$P < 0.005$
	75th percentile of radioactivity		
Total β	9.80 ± 0.59	14.91 ± 1.09	$P < 0.005$
Class II	9.24 ± 0.51	13.06 ± 1.05	$P < 0.005$
Class III	11.00 ± 0.65	15.26 ± 1.12	$P < 0.005$

* Mean distance along nerve (mm) ± standard error at 5 d after labeling, n = 7 nerves for axotomy and 9 for controls.
‡ Differences between axotomy and control values were evaluated using the t test.

We also found that the leading edge of the slow component wave was enriched in the class III isotype in control motor fibers. The behavior of the leading edge of the wave was reflected in the location of the 75th percentile of radioactivity (Table I), which was located more distally for class III than for class II β-tubulin in control motor fibers ($P < 0.05$; t test). In axotomized motor fibers, differences in the locations of the 75th percentiles of radioactivity for class II and III β-tubulin were not statistically significant.

To compare the labeling of cytoskeletal proteins transported in the slow component waves of axotomized and contralateral control fibers, motor neurons were labeled by symmetrically injecting [^{35}S]methionine into both sides of the spinal cord 14 d after unilaterally crushing the sciatic nerve. Animals were sacrificed 5 d after labeling, the L4 and L5 ventral roots from each nerve were combined, and the levels of radioactivity in the NF proteins and tubulins were determined as described in Materials and Methods. These analyses revealed that the levels of radioactivity in class II, class III, and total β-tubulin were significantly greater in axotomized than in contralateral control fibers (Table II). These increases in the labeling of tubulin were accompanied by significant reductions in the labeling of the NF proteins in axotomized motor fibers (Table II). Thus, the increased labeling of the tubulins in the slow component wave did not reflect a global increase in the labeling of transported proteins in axotomized motor neurons.

The quantity Q, which is an index of the amount of transported radioactivity per millimeter of nerve, was compared in axotomized and control motor fibers for class II, class III,

Table II. Labeling of Slowly Transported Proteins

	Axotomy	Control	% Change*	Significance‡
Total β	1,778 ± 294§	586 ± 89	+203	$P < 0.005$
Class II	1,884 ± 204	508 ± 63	+271	$P < 0.005$
Class III	1,840 ± 245	807 ± 108	+128	$P < 0.005$
NF-L	940 ± 125‖	2,732 ± 380	−66	$P < 0.005$
NF-M	510 ± 64	1,437 ± 179	−65	$P < 0.005$
NF-H	626 ± 52	1,446 ± 219	−57	$P < 0.005$

* (Axotomy-Control)/Control × 100%.
‡ Differences between axotomy and control values were evaluated using the *t* test.
§ Amount of radioactivity (mean cpm ± standard error, $n = 7$ pairs of nerves) immunoprecipitated from an aliquot containing 3.75% of the labeled protein in a segment extending 0–25 mm along the nerve 5 d after labeling.
‖ Amount of radioactivity (mean cpm ± standard error, $n = 7$ pairs of nerves) in a cytoskeletal pellet prepared from an aliquot containing 3.75% of the labeled protein in a segment extending 0–25 mm along the nerve 5 d after labeling.

and total β-tubulin. Q was calculated by dividing the mean level of tubulin radioactivity (Table II) by the distance that the 50th percentile of radioactivity had migrated during the 5-d postlabeling interval (Table I). This analysis revealed that Q was greater in axotomized than in control motor fibers, with increases of +102% for total β-tubulin, +160% for class II, and +58% for class III (Table III).

The tubulin contents of axotomized and contralateral control ventral roots were compared using immunoassay. Immunohistochemistry revealed that nearly all of the class II and III β-tubulin in nerve fibers is located within axons (i.e., the same population of motor axons in which transport was examined). To detect potential differences in the spatial distributions of these isotypes, tubulin content was analyzed in 4-mm-long segments harvested from both proximal and distal regions of the ventral roots (i.e., in segments located adjacent to the spinal cord and dorsal root ganglion, respectively). Tubulin levels were analyzed at 5 d (a relatively short postaxotomy interval) and 19 d after axotomy (the same postaxotomy time at which transport was analyzed in these motor fibers; motor neurons were labeled 14 d after axotomy, and transport was analyzed 5 d later). These analyses revealed no significant differences between the tubulin contents of axotomized and control motor fibers at either postaxotomy time (Table IV). In addition, no significant differences were detected between the tubulin contents at proximal and distal levels at either postaxotomy time (Table IV).

Discussion

We have found that the tubulin content of mature motor fibers is relatively insensitive to apparent alterations in the amount of tubulin transported in the slow component wave. We will now consider evidence indicating that this observation is most consistent with the scenario that the slow component wave represents the movement of only a small fraction of the tubulin in mature motor axons.

Table III. Q, the Amount of Radioactivity per Millimeter of Nerve

	Radioactivity*	Distance‡	Q§
	Total β-tubulin		
Control	586 ± 89	6.43 ± 0.51	91
Axotomy	1,778 ± 294	9.67 ± 0.85	184
(% Change)‖	(+203)	(+50)	(+102)
	Class II		
Control	508 ± 63	6.20 ± 0.47	82
Axotomy	1,884 ± 204	8.86 ± 0.81	213
(% Change)	(+271)	(+43)	(+160)
	Class III		
Control	807 ± 108	7.46 ± 0.51	108
Axotomy	1,840 ± 245	10.79 ± 0.96	171
(% Change)	(+128)	(+45)	(+58)

* Amount of radioactivity (mean cpm ± standard error, $n = 7$ pairs of nerves) immunoprecipitated from an aliquot containing 3.75% of the labeled protein in a segment extending 0–25 mm along the nerve 5 d after labeling. These data are also shown in Table II.
‡ The distance (mm) that the 50th percentile of radioactivity has migrated during the 5-d postlabeling interval (Table I).
§ Q, the amount of radioactivity per mm of nerve (cpm/mm), was calculated by dividing the amount of transported radioactivity by the distance of the 50th percentile of radioactivity.
‖ (Axotomy-Control)/Control × 100%.

*Table IV. Protein Content of the Nerve**

		Axotomy	Control	Significance‡
		5 d after axotomy		
Total β	proximal	0.38 ± 0.07	0.46 ± 0.05	NS
	distal	0.48 ± 0.04	0.41 ± 0.08	NS
Class II	proximal	0.90 ± 0.07	0.76 ± 0.11	NS
	distal	0.90 ± 0.14	0.87 ± 0.08	NS
Class III	proximal	0.55 ± 0.04	0.66 ± 0.06	NS
	distal	0.71 ± 0.03	0.66 ± 0.07	NS
		19 d after axotomy		
Total β	proximal	0.75 ± 0.06	0.81 ± 0.12	NS
	distal	0.53 ± 0.10	0.54 ± 0.07	NS
Class II	proximal	1.18 ± 0.13	0.97 ± 0.12	NS
	distal	1.10 ± 0.14	0.94 ± 0.07	NS
Class III	proximal	0.72 ± 0.05	0.80 ± 0.09	NS
	distal	0.84 ± 0.32	0.72 ± 0.06	NS

* Mean ± standard error ($n = 6$) of protein content, expressed in OD units, in proximal (P) and distal (D) 4-mm-long segments of the L5 ventral root. For β-tubulin, 1 OD unit equals 1 μg of protein. For the class II and III isotypes, 1 OD unit corresponds to the amount of that isotype in 1 μg of total β-tubulin in ventral root axons.
‡ Differences in the protein contents of axotomized and control nerves were evaluated using the *t* test.

The Axonal Transport of Class II and III β-tubulin

mAbs specifically directed against the carboxy-terminal sequences of either class II or III β-tubulin were used to immunoprecipitate pulse-labeled tubulin undergoing transport in motor fibers. The isotype specificity of these antibodies was confirmed by demonstrating: (*a*) that they recognized bacterial fusion proteins containing the carboxy-terminal sequences of these isotypes; and (*b*) that immunoprecipitation with these antibodies can be selectively blocked by synthetic peptides bearing the carboxy-terminal sequences of these isotypes. It should also be noted that the abilities of these antibodies to recognize class II and III β-tubulin are not influenced by posttranslational modifications of these isotypes (M. K. Lee, personal communication). We further demonstrated that the immunoprecipitation of class II and III tubulin was quantitative under the conditions of antibody excess used in our analyses (i.e., the amount of labeled tubulin immunoprecipitated was not influenced by alterations in the total amount of tubulin in the reaction mixture).

We have found that both class II and III β-tubulin are associated with the pulse-labeled slow component wave. Studies in which photoactivation was used to examine the movement of fluorescent tubulin in cultured embryonic motor neurons demonstrate that tubulin is transported in the form of microtubule polymers (Reinsch et al., 1991). The similarity between the behavior of fluorescent tubulin in these embryonic axons and the coherent movement of the pulse-labeled tubulin wave in mature motor fibers suggests that tubulin is also transported in its polymeric form in mature motor fibers (Hoffman and Lasek, 1975).

Although the overall distributions of pulse-labeled class II and III β-tubulin were generally similar, we found that the leading edge of the tubulin wave was enriched in the class III isotype in control motor fibers. This confirms the previous observation that the leading edge of the tubulin wave is enriched in an isoelectric variant of β-tubulin (Denoulet et al., 1989), which corresponds to the class III isotype (Edde et al., 1989). Recent studies have shown distinct functional properties of β-tubulin isotypes in insect testis (Hoyle and Raff, 1990). Thus, this difference in the distributions of class II and III β-tubulins supports the view that there may be functional differences among the β-tubulin isotypes present in axons (Joshi and Cleveland, 1989).

Alterations in the Amounts of Tubulin and Neurofilament Proteins Transported in Axotomized Motor Axons

Previous studies have shown that changes in the labeling of transported tubulin and NF protein in axotomized motor fibers (Hoffman and Lasek, 1980; Hoffman et al., 1985*b*; Tashiro and Komiya, 1991) correlate with corresponding alterations in the abundance of mRNAs encoding these proteins in motor neuron cell bodies (Muma et al., 1990; Tetzlaff et al., 1991). This suggests a close correlation between the levels of transported radioactivity and the amounts of protein undergoing transport. Axotomy-induced changes in the expression of specific isotypes of β-tubulin have been previously characterized in peripheral sensory neurons where there is a relatively large increase (sixfold) in the abundance of mRNAs encoding class II β-tubulin (Hoffman and Cleveland, 1988), a much smaller increase (less than twofold) for class III (P. N. Hoffman and M. A. Lopata, unpublished observation), and little, if any, change for classes I and IV (Hoffman and Cleveland, 1988). Such changes are consistent with our finding that labeling was increased more for class II than for class III β-tubulin in axotomized motor fibers.

The Slow Component Wave Represents the Movement of a Substantial Proportion of the Neurofilament Proteins in Mature Motor Axons

The relative amounts of class II and III β-tubulin radioactivity per millimeter of nerve were compared in axotomized and control motor fibers using the quantity Q, which was calculated by dividing the total amount of radioactivity in the nerve by the distance that the 50th percentile of radioactivity had migrated during the 5-d postlabeling interval (see Materials and Methods). The general validity of this approach is supported by previous studies which have shown that alterations in Q resulting from changes in either the rate of NF transport or the labeling of transported NF proteins correlate with alterations in axonal NF content. Since essentially all of the NF protein in axons is in its polymeric form (Morris and Lasek, 1982), the NF content of axons can be measured morphologically by counting the number of NFs.

A reduction in the rate of NF transport (and an increase in Q) induced by systemic intoxication with the neurotoxin β,β′-iminodipropionitrile (IDPN) correlates with an increase in axonal NF content (Chou and Hartmann, 1965; Griffin et al., 1978; Clark et al., 1980; Watson et al., 1989). In contrast, an increase in the transport rate (and a reduction in Q) induced by systemic intoxication with 2,5-hexanedione (2,5-HD) correlates with a decrease in axonal NF content (Monaco et al., 1989*a*,*b*). The abundance of NF mRNAs (and the amount of transported NF radioactivity) is unaltered after systemic intoxication with either IDPN (Parhad et al., 1988) or 2,5-HD (Watson et al., 1991).

Axotomy-induced reductions in the labeling of NF proteins in the slow component wave (Hoffman and Lasek, 1980; Hoffman et al., 1985*b*; Tashiro and Komiya, 1991) correlate with a decrease in the abundance of NF mRNAs in axotomized neurons (Hoffman et al., 1987; Wong and Oblinger, 1987; Goldstein et al., 1988; Muma et al., 1990; Tetzlaff et al., 1991); the rate of NF transport is unaltered (Hoffman et al., 1985*b*). Thus, Q, the amount of NF radioactivity per millimeter of nerve, is reduced after axotomy. Morphological analyses have shown that this decrease in Q correlates with a decline in axonal NF content (by as much as 50% in large-caliber fibers) which starts proximally near the cell body and spreads somatofugally along nerve fibers at a velocity equal to the median rate of the slow component wave (Hoffman et al., 1984). This close correlation between changes in NF transport and alterations in axonal NF content indicate that the slow component wave represents the movement of a substantial proportion of the NF proteins in mature motor fibers (i.e., at least 50% of the NF proteins in large-caliber axons).

Pulse-labeling studies demonstrate that radioactive NF proteins are also retained along motor fibers after passage of the slow component wave (see Fig. 1, Hoffman et al., 1985*a*). It remains controversial whether these NF proteins

have become stationary (Nixon and Logvinenko, 1986) or continue to move at slower rates (Lasek et al., 1992). The observation that NF proteins retained along optic nerve axons disappear after relatively long postlabeling intervals has been interpreted to indicate that these proteins are moving (i.e., at slower rates) rather than stationary (Lasek et al., 1992); alternatively, the disappearance of these proteins could reflect the turnover of stationary NFs.

The Slow Component Wave Represents the Movement of Only a Small Fraction of the Tubulin in Mature Motor Axons

We found that the amounts of radioactivity per millimeter of nerve in both class II and III β-tubulin were greater in axotomized than in control motor fibers (+160% and +58%, respectively). Nevertheless, immunoassay revealed no significant differences in the amounts of these isotypes in axotomized and control motor fibers. How can we account for this discrepancy between changes in the amount of radioactivity per millimeter of nerve and the lack of change in the total tubulin content of these axons measured by immunoassay?

One possibility is that alterations in amino acid precursor pool size have led to changes in incorporation (and levels of transported radioactivity) after axotomy which do not reflect corresponding changes in synthetic rates. For example, if the specific radioactivity of the amino acid precursor pool increased 100% (doubled) after axotomy, then a 50% increase in the actual amount of transported tubulin per millimeter of nerve would be reflected in a 100% increase in the amount of transported radioactivity per millimeter. Although it does not seem very likely, such a possibility is difficult to exclude since we lack direct information concerning the sizes of the precursor pools in either axotomized or control motor neurons.

Another possibility is that our immunoassay was not sensitive enough to detect changes in axonal tubulin content comparable in size with the estimated alterations in the amount of transported tubulin in axotomized motor fibers (i.e., +160% for class II and +58% for class III β-tubulin). This explanation is highly unlikely. A formal estimate of the statistical power of the immunoassay determinations was calculated, based on the control means and the pooled variance, sample size $n = 6$ per group, and the level of significance $\alpha = 0.05$. For the hypothesis that the axotomized means were actually 1.5 times the control means, the power ($1-\beta$) was >0.99 for each of the antibodies assayed. In other words, there was a >99% chance of detecting a change in tubulin levels of 50% or more. Therefore, it is unlikely that there was a change of 50% or more in the tubulin content of axotomized motor fibers.

If we assume that methodological limitations are not responsible for this discrepancy between changes in the amount of radioactivity per millimeter of nerve and the lack of change in the total tubulin content measured by immunoassay, how can we explain this paradox? One possibility is that the increased expression of tubulin simply compensates for a rise in the turnover of tubulin in axotomized motor fibers. This too seems unlikely if we consider the possible significance of increased tubulin expression. The rate of axon elongation during regeneration is comparable with the normal rate of tubulin transport in the SCb component of slow axonal transport (3–5 mm/d) (Lasek and Hoffman, 1976; Wujek and Lasek, 1983). We have found only modest increases in the median transport rates of class II and III β-tubulin in axotomized motor fibers (43 and 45%, respectively). Therefore, it is unlikely that the class II and III β-tubulin synthesized after axotomy participates directly in the elongation of regenerating sprouts located more than several mm from the cell body (i.e., this tubulin would not reach the tips of regenerating sprouts until after elongation was completed) (Hoffman and Lasek, 1980). Nevertheless, this tubulin could participate in the maturation of regenerating sprouts, a process which correlates with an increase in the number of microtubules in the sprouts (Espejo and Alvarez, 1986). In the absence of increased turnover, the enhanced delivery of tubulin to the sprouts (i.e., as the result of increased expression and transport) could facilitate this process.

Our finding that the tubulin content of mature motor fibers is relatively insensitive to alterations in the amount of tubulin moving at the median rate in the slow component wave suggests that these axons contain a second, even larger pool of tubulin which behaves differently from the pulse-labeled tubulin transported in the slow component wave. What is the nature of this second pool of axonal tubulin? As we have already mentioned, the results of pulse-labeling studies indicate that radioactive tubulin delivered by slow axonal transport is retained along axons after passage of the slow component wave (Watson et al., 1990). Although it is a matter of conjecture whether this tubulin becomes stationary or continues to move at slower rates, the possibility that mature axons contain stationary microtubules can be inferred from the results of real-time imaging studies in which photoactivation was used to examine the behavior of fluorescent tubulin in the neurites of cultured adult sensory neurons (Okabe and Hirokawa, 1992). In either case, our findings are most consistent with the scenario that the slow component wave represents the movement of only a small fraction of the tubulin in mature motor fibers.

The Maturation of Developing Axons

The number of axonal microtubules increases during the postnatal maturation of myelinated nerve fibers (Friede and Samorajski, 1970). We propose that the maturation (and possibly the stabilization) of axons during postnatal development reflects the continuous intra-axonal accumulation of microtubule polymers comprised of tubulin originally delivered from the cell body by the slow component wave which either becomes stationary or continues to move at slower rates. According to this model, the fraction transported in the slow component wave accounts for most, if not all of the tubulin in newly formed embryonic axons. In contrast, as axonal tubulin content increases during postnatal development, the fraction transported in the slow component wave represents a declining proportion of the total tubulin content. This model explains how the tubulin content of axons increases as the abundance of tubulin mRNAs (and presumably the amount of tubulin transported in the slow component wave) decreases during postnatal development (Hoffman, 1989; Muma et al., 1991). It also accounts for regional differences in the distribution of axonal microtubules. For example, the number of microtubules is greater at nodes than along internodes of myelinated nerve fibers (Reles and Friede, 1991).

The number of microtubules is also 10-fold greater in the terminal branches than in the stem processes of large-caliber motor fibers (Zenker and Hohberg, 1973).

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References

Banerjee, A., M. C. Roach, K. A. Wall, M. A. Lopata, D. W. Cleveland, and R. F. Luduena. 1988. A monoclonal antibody against the type II isotype of β-tubulin. *J. Biol. Chem.* 263:3029–3034.

Banerjee, A., M. C. Roach, P. Trcka, and R. F. Luduena. 1990. Increased microtubule assembly in bovine brain tubulin lacking the type III isotype of β-tubulin. *J. Biol. Chem.* 265:1794–1799.

Bonner, W. M., and R. A. Laskey. 1974. A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* 46:83–88.

Chou, S.-M., and H. A. Hartmann. 1965. Electron microscopy of focal neuroaxonal lesions produced by 3,3'-iminodipropionitrile (IDPN) in rats. *Acta Neuropathol.* 4:590–603.

Clark, A. W., J. W. Griffin, and D. L. Price. 1980. The axonal pathology in chronic IDPN intoxication. *J. Neuropathol. & Exp. Neurol.* 39:42–55.

Cleveland, D. W. 1987. The multitubulin hypothesis revisited: what have we learned? *J. Cell Biol.* 104:381–383.

de la Vina, S., D. Andreu, F. J. Medrano, J. M. Nieto, and J. M. Andreu. 1988. Tubulin structure probed with antibodies to synthetic peptides. Mapping of three major types of limited proteolysis fragments. *Biochemistry.* 27:5352–5365.

Denoulet, P., G. Filliatreau, B. de Nechaud, F. Gros, and L. Di Giamberardino. 1989. Differential axonal transport of isotubulins in the motor axons of the rat sciatic nerve. *J. Cell Biol.* 108:965–971.

Edde, B., P. Denoulet, B. de Nechaud, A. Koulakoff, Y. Berwald-Netter, and F. Gros. 1989. Posttranslational modifications of tubulin in cultured mouse brain neurons and astroglia. *Biol. Cell.* 65:109–117.

Espejo, F., and J. Alvarez. 1986. Microtubules and calibers in normal and regenerating axons of the sural nerve of the rat. *J. Comp. Neurol.* 250: 65–72.

Friede, R. L., and T. Samorajski. 1970. Axonal caliber related to neurofilaments and microtubules in sciatic nerve fibers of rats and mice. *Anat. Rec.* 167:379–388.

Goldstein, M. E., S. R. Weiss, R. A. Lazzarini, P. S. Shneidman, J. F. Lees, and W. W. Schlaepfer. 1988. mRNA levels of all three neurofilament proteins decline following nerve transaction. *Mol. Brain Res.* 3:287–292.

Greenberg, S. G., and R. J. Lasek. 1988. Neurofilament protein synthesis in DRG neurons decreases more after peripheral axotomy than after central axotomy. *J. Neurosci.* 8:1739–1746.

Griffin, J. W., P. N. Hoffman, A. W. Clark, P. T. Carroll, and D. L. Price. 1978. Slow axonal transport of neurofilament proteins: impairment by iminodipropionitrile administration. *Science (Wash. DC).* 202:633–635.

Hoffman, P. N. 1989. Expression of GAP-43, a rapidly transported growth-associated protein, and class II β tubulin, a slowly transported cytoskeletal protein, are coordinated in regenerating neurons. *J. Neurosci.* 9:893–897.

Hoffman, P. N., and D. W. Cleveland. 1988. Neurofilament and tubulin expression recapitulates the developmental program during axonal regeneration: Induction of a specific β tubulin isotype. *Proc. Natl. Acad. Sci. USA.* 85:4530–4533.

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.

Hoffman, P. N., and R. J. Lasek. 1980. Axonal transport of the cytoskeleton in regenerating motor neurons: constancy and change. *Brain Res.* 202:317–333.

Hoffman, P. N., R. J. Lasek, J. W. Griffin, and D. L. Price. 1983. Slowing of the axonal transport of neurofilament proteins during development. *J. Neurosci.* 3:1694–1700.

Hoffman, P. N., J. W. Griffin, and D. L. Price. 1984. Control of axonal caliber by neurofilament transport. *J. Cell Biol.* 99:705–714.

Hoffman, P. N., J. W. Griffin, B. S. Gold, and D. L. Price. 1985*a*. Slowing of neurofilament transport and the radial growth of developing nerve fibers. *J. Neurosci.* 5:2920–2929.

Hoffman, P. N., G. W. Thompson, J. W. Griffin, and D. L. Price. 1985*b*. Changes in neurofilament transport coincide temporally with alterations in the caliber of axons in regenerating motor fibers. *J. Cell Biol.* 101:1332–1340.

Hoffman, P. N., D. W. Cleveland, J. W. Griffin, P. W. Landes, N. J. Cowan, and D. L. Price. 1987. Neurofilament gene expression: A major determinant of axonal caliber. *Proc. Natl. Acad. Sci. USA.* 84:3472–3476.

Hoyle, H. D., and E. C. Raff. 1990. Two drosophila β tubulin isoforms are not functionally equivalent. *J. Cell Biol.* 111:1009–1026.

Hsu, S. M., L. Raine, and H. Fanger. 1981. The use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques—a comparison between ABC and the unlabeled antibody (PAP) procedures. *J. Histochem. Cytochem.* 29:577–580.

Joshi, H. C., and D. W. Cleveland. 1989. Differential utilization of β tubulin isotypes in differentiating neurites. *J. Cell Biol.* 109:663–673.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.

Lasek, R. J., and P. N. Hoffman. 1976. The neuronal cytoskeleton, axonal transport and axonal growth. *Cold Spring Harbor Conf. Cell Proliferation.* 3:1021–1049.

Lasek, R. J., P. Paggi, and M. J. Katz. 1992. Slow axonal transport mechanisms move neurofilaments relentlessly in mouse optic axons. *J. Cell Biol.* 117:607–616.

Lee, M. K., J. B. Tuttle, L. I. Rebhun, D. W. Cleveland, and A. Frankfurter. 1990. The expression and posttranslational modification of a neuron-specific β-tubulin isotype during chick embryogenesis. *Cell Motil. Cytoskeleton.* 17:118–132.

Lopata, M. A., and D. W. Cleveland. 1987. In vivo microtubules are copolymers of available β-tubulin isotypes: Localization of each of six β-tubulin isotypes using polyclonal antibodies elicited by synthetic peptide antigens. *J. Cell Biol.* 105:1707–1720.

Mitchison, T., and M. Kirschner. 1988. Cytoskeletal dynamics and nerve growth. *Neuron.* 1:761–772.

Monaco, S., L. Autilio-Gambetti, R. J. Lasek, M. J. Katz, and P. Gambetti. 1989*a*. Experimental increase of neurofilament transport rate: decreases in neurofilament number and in axon diameter. *J. Neuropath. & Exp. Neurol.* 48:23–32.

Monaco, S., J. Jacob, H. Jenich, A. Patton, L. Autilio-Gambetti, and P. Gambetti. 1989*b*. Axonal transport of neurofilament is accelerated in peripheral nerve during 2,5-hexanedione intoxication. *Brain Res.* 491:328–334.

Morris, J. R., and R. J. Lasek. 1982. Stable polymers of the axonal cytoskeleton: the axoplasmic ghost. *J. Cell Biol.* 92:192–198.

Muma, N. A., P. N. Hoffman, H. H. Slunt, M. D. Applegate, I. Lieberburg, and D. L. Price. 1990. Alterations in levels of mRNAs coding for neurofilament protein subunits during regeneration. *Exp. Neurol.* 107:230–235.

Muma, N. A., H. H. Slunt, and P. N. Hoffman. 1991. Postnatal increase in neurofilament gene expression and the radial growth of axons. *J. Neurocytol.* 20:844–854.

Nixon, R. A., and K. B. Logvinenko. 1986. Multiple fates of newly synthesized neurofilament proteins: evidence for a stationary neurofilament network distributed nonuniformly along axons of retinal ganglion cell neurons. *J. Cell Biol.* 102:647–659.

Okabe, S., and N. Hirokawa. 1992. Differential behavior of photoactivated microtubules in growing axons of mouse and frog neurons. *J. Cell Biol.* 117:105-120.

Parhad, I. M., E. A. Swedberg, D. I. Hoar, C. A. Krekoski, and A. W. Clark. 1988. Neurofilament gene expression following β,β'-iminodipropionitrile (IDPN) intoxication. *Mol. Brain Res.* 4:293–301.

Reinsch, S. S., T. J. Mitchison, and M. Kirschner. 1991. Microtubule polymer assembly and transport during axonal elongation. *J. Cell Biol.* 115:365–379.

Reles, A., and R. L. Friede. 1991. Axonal cytoskeleton at the nodes of Ranvier. *J. Neurocytol.* 20:450–458.

Sullivan, K. F., and D. W. Cleveland. 1986. Identification of conserved isotype-defining variable region sequences for four vertebrate β tubulin polypeptide classes. *Proc. Natl. Acad. Sci. USA.* 83:4327–4331.

Tashiro, T., and Y. Komiya. 1991. Changes in organization and axonal transport of cytoskeletal proteins during regeneration. *J. Neurochem.* 56:1557–1563.

Tetzlaff, W., S. W. Alexander, F. D. Miller, and M. A. Bisby. 1991. Response of facial and rubrospinal neurons to axotomy: changes in mRNA expression for cytoskeletal proteins and GAP-43. *J. Neurosci.* 11:2528–2544.

Trapp, B. D., Y. Itoyama, N. H. Sternberger, R. H. Quarles, and H. deF. Webster. 1981. Immunocytochemical localization of Po protein in Golgi complex membranes and myelin of developing rat Schwann cells. *J. Cell Biol.* 90:1–6.

Vallee, R. 1982. A taxol-dependent procedure for the isolation of microtubule-associated proteins (MAPs). *J. Cell Biol.* 92:435–442.

Watson, D. F., K. P. Fittro, J. W. Griffin, and P. N. Hoffman. 1989. Phosphorylation-dependent immunoreactivity of neurofilaments increases during axonal maturation and IDPN intoxication. *J. Neurochem.* 53:1818–1829.

Watson, D. F., K. P. Fittro, P. N. Hoffman, and J. W. Griffin. 1991. Phosphorylation-related immunoreactivity and the rate of transport of neurofilaments in chronic 2,5-hexanedione intoxication. *Brain Res.* 539:103–109.

Watson, D. F., P. N. Hoffman, and J. W. Griffin. 1990. The cold stability of microtubules increases during axonal maturation. *J. Neurosci.* 10:3344–3352.

Wong, J., and M. M. Oblinger. 1987. Changes in neurofilament gene expression occur after axotomy of dorsal root ganglion neurons: an *in situ* hybridization study. *Metabolic Brain Disease.* 2:291–303.

Wujek, J. R., and R. J. Lasek. 1983. Correlation of axonal regeneration and slow component b in two branches of a single axon. *J. Neurosci.* 3:243–251.

Zenker, W., and E. Hohberg. 1973. A-α-nerve-fibre: number of neurotubules in the stem fibre and in the terminal branches. *J. Neurocytol.* 2:143–148.