The Cold Stability of Microtubules Increases During Axonal Maturation

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The development of cold-stable microtubules (MTs) was studied in maturing rat sciatic nerves. From 4 to 10 weeks of age, there was a large increase in the proportion of tubulin associated with stable MTs. A greater fraction of axonal tubulin than nonaxonal tubulin was associated with stable MTs. The labeled tubulin remaining behind the peak of slow axonal transport was more highly associated with stable MTs than tubulin in the peak itself. Immunoassay confirmed that the sciatic nerve contains a pool of stable MTs not identical to the peak of tubulin transport. The developmental increase in MT stability is not associated with any increase in the acetylation of tubulin or with alterations in the major MT-associated proteins. One aspect of maturation of the axonal cytoskeleton may involve deposition of tubulin into stable MTs that are either stationary or moving slowly with regard to the peak of transported tubulin.

Microtubules (MTs) are assembled from dimeric tubulin subunits. The reverse process, disassembly of MTs into soluble tubulin, occurs normally in living cells (Schulze and Kirschner, 1986, 1987; Sammak and Borisy, 1988; Lim et al., 1989) and can be promoted by cold, a variety of toxins, and increased intracellular calcium. Many cells contain populations of stable MTs that are resistant to depolymerization (Behnke and Forer, 1967; Pipeleers et al., 1977b; Schulze and Kirschner, 1987). Several different mechanisms may contribute to the physical stability of the MTs that resist depolymerization: the presence of special isoforms of tubulin (Brady et al., 1984; Detrich et al., 1987; Binet and Meininger, 1988), acetylation and detyrosination of tubulin (Piperno and Fuller, 1985; Black and Keyser, 1987; Cambray-Deakin and Burgoyne, 1987; Gundersen et al., 1987; Kreis, 1987; Piperno et al., 1987; Schulze et al., 1987; Bulinski et al., 1988; Sale et al., 1988; Black et al., 1989), and the abundance and post translational modifications of MT-associated proteins (MAPs; Hesketh, 1984; Yamamoto et al., 1985; Job et al., 1987; Ohta et al., 1987; Hoshi et al., 1988).

Mature peripheral nerve axons contain a large population of highly stabilized MTs (Brady et al., 1984; Donoso, 1986; Sahenk and Brady, 1987; Filliatreau et al., 1988; Denoulet et al., 1989). The molecular basis for this stability in axons remains poorly understood; however, proper regulation of the stability of MTs is clearly important for normal maintenance of the axon. For example, drugs such as colchicine or vincristine that reduce MT stability can cause symptomatic neuropathy in humans, marked by distal axonal degeneration (Gottschalk et al., 1968; McLeod and Penny, 1969; Bradley et al., 1970; Riggs et al., 1986; Kuncel et al., 1987).

Relationship of MT stability to axonal maturation

We have investigated the physical stability of intraaxonal MTs in the rat sciatic nerve 3 to 14 weeks of age and particularly in sensory axons coursing peripherally from the L5 dorsal root ganglion. Before 3 weeks of age, the relative abundance of mRNAs for genes encoding distinct isoforms of beta-tubulin changes to the adult pattern in these sensory neurons (Hoffman and Cleveland, 1988). The expanding myelinated axons during this stage of development rapidly accumulate intraaxonal neurofilaments, and there is an associated slowing of transport of neurofilaments and MTs (Komiya, 1980; Hoffman et al., 1983, 1985). It has not been established whether axons develop a large population of stable MTs prior to or in concert with these other aspects of cytoskeletal maturation. Neither has it been established whether the acetylation of tubulin in maturing axons increases in a fashion similar to early development of neurites of cultured cells (Black and Keyser, 1987). The possibility of a change in the types and relative abundance of MAPs is also a hypothetical mechanism for maturation of the axonal cytoskeleton, but information on this point has been lacking.

Our results indicate that there is a large increase in the population of axonal MTs that resist cold depolymerization from 4 to 10 weeks of age. The data further suggest that this is accomplished in part by deposition of transported tubulin into stable MTs that either are stationary or are transported much more slowly than the bulk of slow axonal transport. There is an unexpected decrease in the abundance of acetylated tubulin during maturation. The MAPs in peripheral nerves differ from the MAPs of the brain but do not show any significant maturational change in type or relative abundance from 3 weeks to 3 months of age.

Materials and Methods

Labeling of axonal transport. Experimental procedures were performed in male Sprague–Dawley rats under chloral hydrate anesthesia. Proteins were labeled by microinjection of 100 μ Ci of ³⁵S-methionine dissolved

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in 2 μ l PBS into the L5 dorsal root ganglion via a glass micropipette. For most groups of animals, the interval between injection and death was chosen so that the peak of labeled transported tubulin would be contained in the nerve segment 20–30 mm distal to the ganglion, as illustrated in the transport profile in Figure 1. Because axonal transport slows with maturation, different time intervals were allowed at different ages for labeling: animals labeled at 3 weeks of age were killed 7 d later (4 weeks of age), animals labeled at 6 weeks of age were killed 9 d later (approximately 7 weeks of age), animals labeled at 7 1/2 or 8 1/2 weeks of age were killed 10 d later (9 and 10 weeks of age), and animals labeled at 12 weeks of age were killed 12 d later (14 weeks of age). Another series of animals, labeled at 3 weeks of age, was studied after longer transport intervals (20 or 40 d after labeling) when the bulk of labeled tubulin had passed beyond the segment 20–30 mm from the ganglion.

Preparation of tubulin fractions. Previously reported techniques (Pipileers et al., 1977a; Olmsted, 1981) were adapted to small nerve segments. At 4, 7, 10, and 14 weeks of age, the sciatic nerve was exposed, and the nerve segment from 20 to 30 mm distal to the L5 dorsal root ganglion was excised. The nerve segment was immediately ground with a glass pestle for 30–45 sec in 0.75 ml of an MT-stabilizing solution: 50% glycerol, 5% dimethylsulfoxide (DMSO), 0.1 M piperazine N,N'-ethyl-sulfonic acid (PIPES) buffer (pH, 6.9), 1.0 mM EGTA, and 1.0 mM MgCl₂. The homogenate was then centrifuged at 425,000 $\times$ g for 15 min at 25°C. The supernatant contained tubulin subunits that were not assembled at the time of homogenization. The pellet, containing both stable and cold-labile MTs was resuspended in 0.75 ml 0.1 M PIPES buffer (pH, 6.9) containing EGTA and MgCl₂, but no DMSO or glycerol. The resuspended pellet was held for 1 hr on ice, then centrifuged as before. At this stage, the supernatant contained soluble tubulin released from cold-labile MTs and the pellet contained the stable MT fraction. The pellet was then dissolved in 8 M urea and 5% 2-mercaptoethanol in PBS.

Nonaxonal tubulin. In a different group of animals at 14 weeks of age, nonaxonal tubulin of the segment of sciatic nerve 20–30 mm from the L5 ganglion was labeled by endoneurial injection of ³⁵S-methionine (50 μ Ci in 1 μ l injected over 5 min via a glass micropipette). These experiments took advantage of the fact that mammalian axons lack ribosomes and are incapable of substantial protein synthesis; hence, local labeling preferentially labels proteins of Schwann cells and other nonaxonal elements in nerves. Nerves were subjected to the standard MT fractionation technique 2 hr after labeling; preliminary experiments indicated that much less labeled tubulin could be recovered by 18 hr after labeling.

Determination of tubulin content of fractions. Two different assays of the tubulin content of the nerve fractions were performed on aliquots of the same fractions. The content of labeled transported tubulin was determined by SDS-PAGE, then by cutting the tubulin bands from the gel, digesting them in hydrogen peroxide, and determining ³⁵S content by scintillation counting. Twice-cycled rat brain tubulin was electrophoresed on adjacent lanes to permit identification of the tubulin band. Nonaxonal tubulin, labeled by local injection into the nerve, was analyzed in the same fashion as axonal tubulin. In some additional experiments, locally labeled tubulin or axonally transported labeled tubulin was subjected to a preliminary immunoprecipitation with an excess of polyclonal rabbit anti-tubulin serum (Sigma) bound to Affi-Gel beads (BioRad). The beads were repeatedly washed with PBS, then the bound tubulin was eluted with SDS, urea, and 2-mercaptoethanol and electrophoresed, and the ³⁵S content of the tubulin band was counted.

For all experiments with labeled tubulin, the total tubulin cpm was computed for each individual nerve as the sum of the cpm recovered from the tubulin band for all 3 fractions of that nerve. The cpm in the tubulin band from each fraction was then expressed as a percentage of the total tubulin cpm in that individual nerve. Means and SEs were then calculated from these percentages for $n = 8$ nerves per group.

The total tubulin content of each fraction was independently determined by an enzyme-linked immunosorbent assay in microtiter wells. Wells were treated for 30 min with a 0.1% solution of polylysine, then washed extensively 0.1% TWEEN-20 in PBS after this step and each subsequent step. Aliquots of the nerve fractions were diluted 10- to 200-fold in 0.8 M urea with 0.5% 2-mercaptoethanol in PBS, and nerve proteins were allowed to adsorb to the plate overnight at 4°C. Excess protein-binding sites were blocked with 3% horse serum for 1 hr. Primary alpha-tubulin and beta-tubulin monoclonal antibodies, obtained from Amersham as ascites fluid, were diluted 1:2000 in PBS before use. Wells were exposed to primary antibody for 2.5 hr followed by biotinyl-

Figure 1. Profile of axonal transport 7 d after labeling the L5 ganglion in a 3-week-old rat. Gel electrophoresis of 5-mm nerve segments was performed with fluorographic detection of labeled proteins. Tubulin labeling (*TUB*) is most intense in the segments 20–30 mm from the ganglion; these segments were used in subsequent experiments on MT stability (arrow). The triplet neurofilament proteins (*NF-H*, *-M*, and *-L*) have just reached the segment 20–25 mm from the ganglion; actin (*ACT*) shows a distribution similar to tubulin.

lated equine anti-mouse IgG and subsequently with avidin-biotin-peroxidase complexes (Vector Labs). The amount of bound peroxidase was detected by enzymatic reaction with hydrogen peroxide and *o*-phenylene-diamine in citrate buffer (pH, 5.0). The reaction was stopped by addition of 5N H₂SO₄. Optical density was determined at 492 nm with an automatic Titertek ELISA reader.

Duplicate wells were prepared for each sample for each antibody. Rat brain tubulin prepared by 2 cycles of polymerization was used as a standard; concentrations of 0, 25, 50, and 100 ng/ml tubulin monomer were run in parallel with experimental samples. Before final determination of tubulin content, preliminary immunoassay on serial dilutions of nerve samples was performed to identify dilutions which corresponded to 25–50 ng/ml and to verify that optical density was within a linear range for serial dilution of nerve. Addition of tubulin as an internal standard demonstrated that the presence of nerve homogenate caused no interference with the detection of tubulin (Fig. 2).

No significant differences between alpha- and beta-tubulin content were identified for any group of samples. Therefore, in subsequent data analysis, the sum of alpha- and beta-tubulin for each individual sample was used; the SE for each group was less for this sum than for either monomer considered alone.

Immunoblots. Segments (10 mm) of sciatic nerve were removed from 3- or 12-week-old rats ($n = 6$ rats of both ages) and homogenized in 400 μ l 8 M urea with 5% 2-mercaptoethanol in PBS. SDS was added to 40- μ l aliquots to a final concentration of 1%, and the solutions were boiled for 2 min, then centrifuged at 16,000 $\times$ g. Pooled aliquots from 2 nerves were loaded on each lane of a 3–10% polyacrylamide gel. After electrophoresis, the proteins were transferred to nitrocellulose overnight (Towbin et al., 1979). The nitrocellulose was blocked with 0.5% milk in PBS, then exposed to 1 of the following antibodies: Amersham alpha-tubulin antibody, antibody 6-11-B to acetylated alpha-tubulin (a gift from Dr. G. Piperno), MAP2-C monoclonal antibody (a gift from Dr. A. Matus), or Tau-2 antibody (a gift from Dr. L. Binder), each at a dilution in ascites fluid of 1:2000. Blots were extensively washed, then

Figure 2. Tubulin immunoassay. Duplicate wells and least-squares regression lines are shown. *Top*, Absorbance as a function of concentration of purified tubulin standard fit a straight line for alpha- and beta-tubulin monoclonal antibodies. *Bottom*, Dilutions of nerve homogenates gave a linear relationship of optical density to concentration (solid squares). Addition of a known concentration of purified tubulin standard (STD) to the nerve homogenates (open circles) showed strictly additive effects, that is, no interference of nerve homogenate proteins with detection of tubulin. Data illustrated are for the beta-tubulin antibody.

the bound primary antibody was detected with goat anti-mouse secondary antibody and peroxidase-antiperoxidase complexes.

Preparation of MT and associated proteins from the PNS. Bilateral sciatic nerves from hip to knee were rapidly removed from 10 rats at 3 weeks of age or 6 rats at 12 weeks of age, frozen at -70°C , then thawed to partially disrupt stable MT. Other preparations were made from fresh rat brain. Tissue was homogenized in 1 vol 0.1 M PIPES buffer (pH, 6.9) with 1 mM EGTA, 1 mM MgCl_2 , 1 mM phenylmethylsulfonyl fluoride (PMSF), and 2 $\mu\text{g}/\text{ml}$ leupeptin. The remainder of the MT isolation procedure was that of Vallee (1982), including washing with 0.35 M NaCl in the presence of 20 μM taxol to partially separate MAPs from the MT.

Results

Characterization of tubulin fractions

Electron micrographs of glutaraldehyde-fixed pellets revealed abundant profiles of MTs in the final stable MT fraction. In some instances, these MTs appeared to be embedded in fragments of axonal cytoskeleton with associated neurofilaments (Fig. 3A). Myelin and the axolemma were disrupted by the procedure. There was a characteristic composition of the fractions apparent on either Coomassie blue staining or on fluo-

Figure 3. Characterization of tubulin fractions. *A*, Electron micrograph of the final stable MT pellet. A typical fragment of axonal cytoskeleton is shown. MTs with diameters of 24–25 nm are abundant; their orientation is more variable than in intact axons. *B*, Coomassie blue-stained gel of tubulin fractions from a single nerve in a rat at 4 weeks of age. U, fraction containing unassembly subunits; L, fraction with tubulin from labile MTs; S, fraction with stable MTs. Dashes indicate MW markers: 92, 68, 45 and 25 kDa. *C*, Fluorogram of the same gel (transport labeled 7 d before harvest). Arrow indicates the mobility of tubulin standard. A substantial portion of the labeled tubulin appears in each fraction.

rography of gels (Fig. 3B,C); for example, actin was most abundant in the initial supernatant (fraction with unassembly subunits), and neurofilament proteins were nearly all contained in the final pellet (fraction with stable MT).

Axonal maturation and MT stability

There was a significant maturational increase in stability of MTs from 4 to 14 weeks of age, especially in the interval of 4–10 weeks of age. Immunoassay of total tubulin contained in the nerve indicated an increase in the absolute amount of tubulin in each fraction; however, a much greater proportion of the tubulin was associated with stable MTs by 10 weeks of age (Fig. 4). The labile MT population by immunoassay was small at each age, and consequently, the increase in the proportion of stable MTs by ELISA was accompanied by a parallel decrease in the proportion of tubulin in the unassembly fraction.

A large maturational increase in the proportion of tubulin associated with stable MTs was also seen when labeled transported tubulin was assayed (Fig. 4), though there was a systematic difference in the proportions with the 2 methods of tubulin quantitation (see below). At 4 and 7 weeks of age, a significant portion of the labeled transported tubulin was recovered with the labile MT fraction (Fig. 4). The absolute amount of radioactive tubulin did not change significantly with age: $15,375 \pm 3635$ cpm at 4 weeks and $14,155 \pm 1795$ cpm at 10 weeks of age.

The proportion of radioactive tubulin in the form of stable and labile MTs was not critically affected by the time allowed for transport within a few days (though very long intervals so that the peak of tubulin transport passed beyond the assayed segment had a considerable effect, as described below). For example, for rats labeled at 8 weeks of age, transport intervals of 5 or 10 d gave indistinguishable results for tubulin fractions: $52.5 \pm 4.6\%$ and $44.8 \pm 4.8\%$ of tubulin in the stable MT fractions, respectively ($n = 8$, $0.2 > p > 0.1$ by *t*-test).

Figure 4. Maturation change in stability properties of MTs. MT fractions were prepared from sciatic nerves containing the peak of labeled transport. Tubulin content was measured either by immunoassay (open bars) or by counting ^{35}S from gel bands (solid bars). Results are expressed as a percentage of the total tubulin recovered in the 3 fractions for immunoassay or as a percentage of the total cpm recovered in the tubulin bands from the 3 fractions. Bars indicate SE for $n = 8$ nerves. *Top*, Labile MT fraction. There is a maturational decrease in the content of labeled transported tubulin in the labile fraction. At each age, the transported labeled tubulin was more highly associated with labile MTs than the total nerve tubulin by immunoassay. *Bottom*, Stable MT fraction. There is a maturational increase in the proportion of tubulin associated with stable MTs. At each age, the transported labeled tubulin was less associated with stable MTs than the total tubulin by immunoassay.

Comparison of axonal and nonaxonal tubulin pools

At each age studied, the proportion of tubulin in the labile MT fraction was smaller by immunoassay than that obtained by analysis of the labeled transported tubulin, and the stable MT fraction was larger by immunoassay. These results suggested that there is a pool of stable MTs in the sciatic nerve that are distinct from the population of labeled MTs in the peak of slow axonal transport. In principle, this MT pool might be extra- or intraaxonal.

The stability of nonaxonal MTs was assayed by local labeling of the nonaxonal structures of nerves, followed by standard fractionation and gel electrophoresis. Because it was uncertain whether other labeled proteins at the same apparent MW as tubulin might be prominent in the locally labeled material, a portion of each fraction was subjected to immunoprecipitation of tubulin before electrophoresis. The proportion of locally labeled nonaxonal tubulin associated with stable MTs was much less than the stable MT fraction of transported tubulin (Fig. 5).

Figure 5. Comparison of axonal versus nonaxonal tubulin. Tubulin fractions from mature nerves (14 weeks of age) containing labeled axonal transport were compared to 14-week-old nerves labeled by local endoneurial injection of ^{35}S -methionine. Percentage of the total tubulin cpm in each of the 3 fractions was determined for $n = 8$ nerves; the mean $\pm$ SEM of this percentage is shown. *Total*, Standard gel electrophoresis and counting of the tubulin bands; no immunoprecipitation of fractions prior to electrophoresis. *Immunoprecipitation*, Tubulin from each fraction was precipitated with rabbit polyclonal anti-tubulin antibody bound to agarose beads prior to electrophoresis. The proportion of axonal stable MTs was greater than the nonaxonal stable MT fraction. There was no significant difference in the percentages with or without immunoprecipitation prior to electrophoresis.

This difference was apparent whether the entire labeled nerve fraction or the immunoprecipitated tubulin cpm from each fraction was analyzed (Figure 5). Hence, the local labeling experiments give no evidence for any large pool of extraaxonal stable MTs to explain the observed differences between the peak of labeled transport and the total tubulin in the nerve, and they suggest that there might be different intraaxonal pools of tubulin.

MT stability and the tubulin remaining behind the peak of slow axonal transport

The presence of an intraaxonal pool of tubulin that is highly associated with stable MTs was directly demonstrated by experiments that examined the labeled tubulin remaining in the sciatic nerve after the peak of slow axonal transport had passed. Three groups of animals were labeled at 3 weeks of age and allowed to survive for various periods (7, 20, or 40 d) before the nerves were removed and the tubulin fractionated. The association of labeled transported tubulin with stable MTs increased substantially with increasing survival interval after labeling (Fig. 6). Because the transported labeled tubulin gradually moves beyond the assayed segment, less total tubulin radioactivity was recovered at 20 d and much less at 40 d after injection: $11,525 \pm 560$ cpm (mean $\pm$ SEM) and 1595 ± 175 cpm, respectively, versus the peak (7 d) value of $15,375 \pm 3635$ cpm.

The high degree of association of residual tubulin with stable MTs was not simply an effect of maturation of the nerve during the transport interval. Animals that were the same age (9 weeks) at death but labeled at 7½ instead of 3 weeks of age, so that the peak of labeled tubulin would be present in the nerve, showed a significantly smaller proportion of tubulin in the stable MT fraction (Fig. 7) compared to the residual tubulin. At 9 weeks

Figure 6. Effect of transport interval on MT stability. Groups of rats labeled at 3 weeks of age were allowed to transport for 7 d (sufficient for the peak of tubulin to reach the assayed segment) or for 20 or 40 d (assayed segment contained residual transported tubulin at these times). The proportion of tubulin recovered in the labile MT fraction decreased, and the proportion in the stable MT fraction increased, with greater times after labeling. Mean and SEM for $n = 8$ nerves are illustrated.

Figure 7. Comparison of the transported peak and residual tubulin in rats of the same age. Nerve segments were harvested at 9 weeks of age from rats labeled 10 d before (segment containing the peak of tubulin transport) or 40 d before (containing residual or very slowly transported tubulin; data redrawn from Fig. 6 for comparison). The residual tubulin is more highly associated with stable MTs and less with labile MTs than the peak of transport ($n = 8$ nerves each group; $p < 0.05$ by 2-tailed t -test for the labile MT and the stable MT differences).

of age, there was also a greater proportion of the peak of tubulin in association with labile MTs when compared to the residual tubulin (Fig. 7).

Acetylated tubulin immunoreactivity

The MTs in the stable fraction were enriched in acetylated tubulin immunoreactivity as compared to the labile MTs. Quantitative immunoassay was performed on duplicate sets of wells plated with tubulin fractions from 10-week-old nerves ($n = 8$) employing either the Amersham alpha-tubulin antibody or the acetylated tubulin antibody 6-11-B. Each antibody was used at saturating concentration, and all secondary steps were carried out in parallel (except that twice as much time was allowed for development of color for the acetylated tubulin wells). Under these conditions, the optical density for the acetylated tubulin in the stable MT fraction was $36.5 \pm 2.1\%$ of the optical density for the total alpha-tubulin, versus only $6.5 \pm 2.0\%$ for the labile MT fraction. The "unassembled" fraction gave an intermediate value of $21.7 \pm 3.2\%$. Expressed another way, there was a 5.6-fold enrichment in the acetylated tubulin in the stable MTs as compared to the labile MT fraction, and the stable MT fraction accounted for more than 70% of the total acetylated tubulin immunoreactivity.

Although the stable MT fraction was enriched in acetylated tubulin immunoreactivity, the developmental increase in MT stability was not mediated by an increase in the abundance of acetylated tubulin. Total immunoreactivity of nerve MTs toward an antibody against acetylated alpha-tubulin showed a decrease from 3 weeks to 3 months of age (Fig. 8). This decrease was apparent with immunoblots or with quantitative immunoassay. The decrease was a modest 29% when total immunoreactivity per unit length of nerve was considered; however, when the immunoreactivity was adjusted for the greater total tubulin content (or the greater total protein content), the relative abundance of the acetylated epitope at 3 months of age was only 20% of the value at 3 weeks of age.

Immunoblots revealed that more than 1 band in the region of tubulin reacts with the antibody 6-11-B. The higher apparent

MW band comigrates with the alpha-tubulin band as identified by the Amersham alpha-tubulin antibody. A second band with lower apparent MW showed the most abundant reaction with the antibody to the acetylated epitope at 3 weeks of age. This band was much less prominent by 3 months of age. A fainter band between these 2 major bands was just visible at either age in heavily loaded lanes.

Although suitable standards for exact quantitation of acetylated alpha-tubulin from the rat are not available, immunoblots suggest that acetylated forms are only a small proportion of the total tubulin. The immunoblots in Figure 8 were performed strictly in parallel with strips of nitrocellulose transferred from the same gel, with the same secondary reagents, and with the primary antibodies at saturating concentrations. The reaction with the acetylated tubulin antibody was much fainter than with the alpha-tubulin antibody, despite the fact that the lanes for the acetylated antibody were loaded with 10 times the total amount of nerve homogenate and developed for 5 times as long at the final enzymatic detection step.

MAPs of peripheral nerve

Silver- or Coomassie blue-stained gels confirmed that tubulin was the most abundant protein in the taxol-stabilized MTs from peripheral nerves. Of the MAPs released by extraction with 0.35 M NaCl (in the presence of 20 μ M taxol), the most abundant were 2 proteins migrating as broad bands between 60 and 65 kDa, probably tau proteins (Fig. 9). One band at approximately 140 kDa largely remained associated with MTs in the presence of 0.35 M NaCl; the other MAPs, including bands at 120 and 85 kDa, were efficiently extracted by this solution. The identity of these bands is not yet confirmed by other techniques; they are "microtubule-associated" in the operational sense of selective pelleting with assembled taxol-stabilized MTs after initial solubilization from the nerve.

These MAPs from nerves differed in relative abundance from MAPs isolated from the brain by the same technique. High-MW MAPs from the sciatic nerve also included 2 closely spaced

Figure 8. Immunoblots comparing acetylated tubulin (antibody 6-11-B) and total alpha-tubulin (antibody from Amersham) at 3 or 12 weeks of age. Each lane was loaded with nerve homogenate in SDS from a different animal. Lanes for 6-11-B were loaded with material from a total of 1 mm of nerve; those for the alpha-tubulin antibody were loaded with material from a total of 0.1 mm of nerve. There was more total immunoreactivity toward antibody 6-11-B at 3 weeks of age, despite the fact that there was much less total tubulin. The major band reacting with 6-11-B at 3 weeks of age had a lower apparent MW than the bulk of alpha-tubulin.

bands with mobility similar to MAP2 from the rat brain and reactive with the MAP2-C monoclonal antibody on immunoblots (data not shown). Two bands from sciatic nerves with MWs of approximately 60–65 kDa reacted with the Tau-2 antibody, which reacts with both the nonphosphorylated and the phosphorylated forms of tau (data not shown).

There was no change in the relative amount or the electrophoretic mobility of any of the prominent MAP bands during maturation from 3 weeks to 3 months of age. There was a developmental increase in the abundance of these bands of roughly proportional to the increase in the total tubulin content. No shift in tau or MAP2 electrophoretic mobility by immunoblot was apparent with maturation from 3 to 12 weeks of age.

Discussion

Stable MT and intraaxonal pools of tubulin

Between 4 and 10 weeks of age, there is a large increase in the population of cold-stable MTs in the rat sciatic nerve, with a modest further increase from 10 to 14 weeks of age (only labeled transported tubulin was analyzed at 14 weeks). This increase is considerably later than the maturational increase in MT stability in the rat cerebellum (Faivre et al., 1985). The increase in MT stability in the sciatic nerve is apparent with immunoassay of total nerve tubulin or with analysis restricted to labeled axonally transported tubulin. Although both techniques show a maturational increase in MT stability, the results are not identical, even when performed on aliquots of the same solutions.

At each age studied, a greater proportion of tubulin was present as stable MTs by immunoassay as compared to analysis of labeled transported tubulin. This suggests that the nerve as a

Figure 9. SDS gel of MAPs from sciatic nerve stained with Coomassie blue followed by silver. Taxol-stabilized MTs were prepared from sciatic nerves at 3 weeks (3) or 12 weeks (12) of age. Nerve MTs were then extracted with 0.35 M NaCl and centrifuged to give soluble MAPs in the supernatant. Positions of MAP2 and alpha- and beta-tubulin (from brain MTs) and of MW markers are indicated. The most prominent MAPs are at 60–65 kDa, possibly tau proteins. Other reproducible bands have MW of approximately 140, 120, and 85 kDa.

whole contains a larger population of stable MTs than does the peak of slow axonal transport of tubulin. Assay of locally labeled nonaxonal MTs indicates that the tubulin in these nonaxonal structures of nerves is less highly associated with stable MTs than with axonal tubulin, and therefore, nonaxonal sources of tubulin cannot represent the highly stabilized MTs observed by immunoassay. It therefore appears that different intraaxonal pools of tubulin are associated with MTs of different stability.

The sciatic nerve contains motor axons as well as sensory axons. If a very high proportion of the tubulin in motor axons were in the form of stable MTs, this might account for the difference between immunoassay of total nerve tubulin and the labeled transported tubulin in sensory axons. Filliatreau and colleagues (1988), employing techniques broadly similar to those employed here, found 68–75% of labeled tubulin in an insoluble MT fraction from motor axons that were 13 weeks old at harvest. This is quite comparable to the 68% (average of the determinations with and without immunoprecipitation) of labeled tubulin found for the 14-week-old sensory axons in the present study. Hence, it seems unlikely that mature motor axons contain a much larger population of stable MTs than sensory axons. Preliminary determinations in this laboratory of labeled tubulin fractions from L5 motor axons at 10 weeks of age also give values similar to those observed for sensory axons (P. N. Hoffman, unpublished observations).

The solubility of the labeled tubulin that moves ahead of the peak of transported tubulin has also been examined, and a smaller amount of this rapidly moving tubulin was found in association with insoluble polymers (Filliatreau et al., 1988). Therefore, the labeled tubulin moving ahead of the main peak of tubulin transport is not likely to represent a pool of highly stable MTs.

A more direct demonstration of an intraaxonal pool of tubulin highly associated with stable MTs was obtained in this study by analysis of the residual labeled tubulin after the peak of

labeled transported tubulin had passed. The high degree of association of this residual labeled tubulin with stable MTs suggests that there is deposition of some tubulin into MTs that are stable and are either stationary or moving at a rate much slower than that of the bulk of slow axonal transport. This hypothesis, that axons contain a stationary (or quasi-stationary) population of MTs that slowly exchange with passing transported material, has a parallel in previous observations of stationary (or nearly stationary) labeled neurofilaments within the distal optic nerve axons of the mouse (Nixon and Logvinenko, 1986). The possibility that labeled transported cytoskeletal proteins may exchange with proteins already resident in the axoplasm has also been proposed as a possible mechanism for the slowing of the rate of transport of labeled proteins at increasing distances from the cell bodies of motor axons (Watson et al., 1989).

Relationship of tubulin fractions to tubulin state in vivo

The fractionation described in this study employs highly artificial conditions, so the correspondence of these fractions to the form of tubulin *in vivo* is not precise. The technique is likely to underestimate the population of stable MTs. The initial homogenization solution lacks GTP and therefore should not promote fresh polymerization of subunits into MT. Furthermore, there is rapid dilution of the axoplasmic volume by roughly 100-fold during the homogenization, which should further disfavor fresh polymerization. On the other hand, the vigorous homogenization required to disperse peripheral nerves may mechanically disrupt some MTs that would otherwise be stable to cold exposure. Because acetylated tubulin generally occurs in stable MTs (Piperno and Fuller, 1985; Piperno et al., 1987; Bulinski et al., 1988), the presence of some acetylated tubulin immunoreactivity in the "unassembled" tubulin fraction suggests that some stable MTs were disrupted during fractionation. Nonetheless, the 5-fold enrichment of acetylated tubulin in the stable MTs as compared to the labile MT fraction indicates that there is some correlation between the fractions produced in these experiments and the state of the tubulin and MTs *in situ*. Electron microscopy also confirms that intact MT segments are abundant in the stable MT pellet. It is not known whether this fraction contains insoluble tubulin in the form of oligomers or structures other than MTs.

Another methodologic consideration is the use of 1-dimensional gel electrophoresis to separate labeled tubulin from other proteins. This strategy depends on the simple composition of the labeled material carried by slow axonal transport in peripheral nerves. Prior 2-dimensional gel electrophoresis and peptide mapping of labeled transported material from motor axons have shown that tubulin is indeed the most abundant labeled protein at this electrophoretic mobility (Brady et al., 1984; Tashiro et al., 1984; Tashiro and Komiya, 1989). Two results reported here further indicate that no other labeled polypeptides with the same electrophoretic mobility as tubulin unduly influence our results. First, there is a prominent maturational increase in MT stability by immunoassay, which does not depend on electrophoretic separation. Second, the proportion of labeled tubulin in the 3 fractions was the same for lanes loaded with entire fractions or loaded with tubulin immunoprecipitated from the fractions before electrophoresis.

Mechanisms of MT stability

The present studies investigated 2 molecular mechanisms that might account for increased MT stability with age. The acety-

lation of alpha-tubulin, as determined by immunoassay, did not increase; instead, there was an absolute decrease from 3 weeks to 3 months of age. This suggests that acetylation of alpha-tubulin is not an important event in the stabilization of mature axonal MTs, in contrast to the case of neurites extending from cultured neurons (Black and Keyser, 1987; Black et al., 1989). It is valid to conclude that immunoreactive acetylated tubulin does not increase in parallel with the increase in MT stability, whether only the band at the same mobility as alpha-tubulin is considered, or whether both bands (including the one at lower apparent MW) are considered together.

The reaction of the acetylated tubulin antibody with a band below the usual mobility of alpha-tubulin in the 3-week-old nerve was unexpected. Because of the specificity of this antibody for acetylated alpha-tubulin in other systems (Piperno and Fuller, 1985; Piperno et al., 1987; Sale et al., 1988), it seems likely that a posttranslational modification of acetylated alpha-tubulin has led to the altered electrophoretic mobility. One alternative is that limited proteolysis has generated a fragment with slightly lower molecular mass but with preserved immunoreactivity to 6-11-B. This seems less likely because no similar band reacted with the alpha-tubulin monoclonal antibody, even though aliquots of the same nerve sample, electrophoresed on different lanes of the same gel, were used for this experiment. Other alternatives include the presence of a novel genetically determined isoform of alpha-tubulin, cross-reaction of the primary antibody with a protein other than tubulin, or cross-reaction of the secondary antibody used for detection with a nerve component, this cross-reaction becoming apparent only with the longer times for enzymatic reaction employed with the acetylated tubulin antibody.

Changes in abundance, types, or posttranslational modifications of MAPs can affect the stability of MTs (Hesketh, 1984; Yamamoto et al., 1985; Job et al., 1987; Baudier and Cole, 1988; Hoshi et al., 1988), and there are changes in MAPs of the PNS during embryonic development (Tucker et al., 1988). Hypothetically, changes in MAPs during the period of maturation from 3 to 12 weeks of age might exert a major influence on MT stability; however, MAPs isolated from nerves did not show any prominent maturational change. Posttranslational modifications of MAPs that do not change their electrophoretic mobilities would not have been detected with the current method and remain as hypothetical mechanisms for maturational increases in stability. The technique employed may also isolate some subset of the total MAPs in the nerves. In particular, the MAPs associated with labile MTs are likely to be overrepresented, because the MAP preparation depends on solubilizing the MTs and their MAPs at the initial step. The use of a freeze/thaw cycle before homogenization was intended to partially disrupt the stable MTs and release their MAPs; however, the exact contribution of stable and labile MTs to the MAP preparations remains unknown. The "microtubule-associated" proteins in peripheral nerves, operationally defined, include MAPs different from the brain. Localization of these proteins *in situ* and definition of their relationship to established MAPs remain to be accomplished.

Adaptive significance of stable MT

Mature axons contain a large population of highly stable MT (Brady et al., 1984; Donoso, 1986; Sahenk and Brady, 1987; Filliatreau et al., 1988) as compared to other cells (Behnke and Forer, 1967; Pipeleers et al., 1977b; Schulze and Kirschner,

1987; Sammak and Borisy, 1988). The function of these stable MTs is not clearly understood, but some hypotheses merit consideration. The axon must remain as an extended cell process throughout the life of an animal, and stable MTs that are stationary or moving very slowly may serve to stabilize axonal structure. Stable MTs might also be important mechanical substrates for fast axonal transport. The distal axon remains critically dependent on a continuing supply of materials via fast axonal transport, and it is likely that the cell body requires uninterrupted retrograde transport to maintain normal function. Indeed, a number of drugs that destabilize MTs are known to interfere with fast axonal transport: colchicine (Kreutzberg, 1969), vincristine (Green et al., 1977), maytansine (Donoso et al., 1978), and nocodazole (Samson et al., 1979). One possible basis for the disruption of axonal transport by agents that alter MT stability has been suggested by recent work showing that 2 of the intracellular "motors" that are likely to drive axonal transport depend on a physical interaction with assembled MTs: kinesin (Vale et al., 1985a, b) and MAP1C (Paschal and Vallee, 1987; Paschal et al., 1987). Hence, the presence of a large population of highly stabilized axonal MTs may be a safeguard to ensure uninterrupted anterograde and retrograde fast axonal transport.

An alternative view of the increase in MT stability with axonal maturation is that immature axons must transport a larger proportion of tubulin in the form of subunits or readily depolymerizable MTs in order to support distal axonal elongation (Bamburg et al., 1986) or other developmental changes that require a high degree of plasticity of the axonal cytoskeleton. In this case, the later accumulation of stable MTs that move slowly (or do not move) may reflect an economy of metabolic effort in mature axons, which no longer require such an abundance of tubulin subunits to reach their distal regions.

The present observations give little insight into the physical form of the tubulin undergoing slow axonal transport. A measurable amount of transported tubulin was associated with all 3 fractions, unassembled subunits, labile MTs and stable MTs for all the conditions studied. The time resolution of such experiments is not sufficient to determine which forms of tubulin are undergoing transport at any instant, because interconversion between subunits and labile MTs could be much more rapid than axonal transport over the macroscopic distances studied. Clarification of the relationship of the highly stabilized form of MTs in axons to the varied biologic functions of MTs poses a major challenge for future investigation.

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