The Distribution of Tau in the Mammalian Central Nervous System

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ABSTRACT We have determined the biochemical and immunocytochemical localization of the heterogeneous microtubule-associated protein tau using a monoclonal antibody that binds to all of the tau polypeptides in both bovine and rat brain. Using immunoblot assays and competitive enzyme-linked immunosorbent assays, we have shown tau to be more abundant in bovine white matter extracts and microtubules than in extracts and microtubules from an enriched gray matter region of the brain. On a per mole basis, twice-cycled microtubules from white matter contained three times more tau than did twice-cycled microtubules from gray matter. Immunohistochemical studies that compared the localization of tau with that of MAP2 and tubulin demonstrated that tau was restricted to axons, extending the results of the biochemical studies. Tau localization was not observed in glia, which indicated that, at least in brain, tau is neuron specific. These observations indicate that tau may help define a subpopulation of microtubules that is restricted to axons. Furthermore, the monoclonal antibody described in this report should prove very useful to investigators studying axonal sprouting and growth because it is an exclusive axonal marker.

Of the numerous microtubule-associated proteins (MAPs)[1] (40) that co-purify with brain tubulin through repetitive cycles of temperature-dependent assembly and disassembly, MAP2 and tau have been most extensively studied. In particular, the high molecular weight protein MAP2 (M_r > 300,000) has received considerable attention. Immunocytochemical studies have shown that MAP2 does not localize in most glia but is highly concentrated in neuronal cell bodies and dendrites (2, 9, 13, 21, 32). Most axons do not exhibit anti-MAP2 staining, in agreement with axoplasmic transport studies on retinal ganglion cells, which have failed to detect transport of MAP2 (5, 46). However, some axons contain detectable amounts of MAP2. Light MAP2 immunoreactivity is apparent in certain axonal subpopulations in the brain proper (9, 17), whereas some axons of the peripheral nervous system stain intensely with monoclonal antibodies to MAP2 (34). These observations are supported by biochemical studies on microtubules purified from white matter, which demonstrate the presence of small amounts of MAP2 (48). However, with these notable exceptions, the majority of axons display little detectable MAP2, indicating that MAP2 is primarily a dendritic protein (2, 9, 13, 17, 21, 34).

MAP2 is a high molecular weight, heat stable doublet of polypeptides (24), and tau is also heat stable but of lower molecular weight (M_r 52,000–68,000) (11). Tau is more electrophoretically heterogeneous. Depending upon the species under study, tau associated with twice-cycled microtubules has been observed as a single electrophoretic species (guinea pig), three sharply resolved polypeptides (mouse), four poorly resolved polypeptides (rat) (16), or four sharply resolved polypeptides (bovine) (11, 15). Early studies (10, 11) demonstrated that, although electrophoretically heterogeneous, the tau polypeptides have nearly identical peptide maps, leading to much speculation as to the nature and purpose of tau's diversity. Both MAP2 and tau stoichiometrically stimulate microtubule assembly in vitro (11, 24, 28, 33, 41, 51) and bind along the entire length of the polymer (reference 24 and Binder, L. I., and L. I. Rebhun, manuscript in preparation).

During brain maturation, MAP2 and tau have been shown to undergo changes in electrophoretic mobility, indicating either the activation of new genes or the occurrence of a series of posttranslational modifications (4, 7, 15, 16, 18, 30). Although the functional implications of these alterations are not known, the immature forms of MAP2 and tau are less efficient

[1] *Abbreviations used in this paper:* ELISA, enzyme-linked immunosorbent assay; MAP, microtubule-associated protein; TBS, 50 mM Tris-HCl, 130 mM NaCl, 5 mM KCl, 5 mM $MgSO_4$, 1 mM EGTA, 0.05% NaN_3, pH 7.6.

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in promoting microtubule assembly in vitro than are their corresponding adult forms (16). The latter result suggests that microtubules in immature brain are less stable than those found in the adult. It is interesting to note that these developmental changes in MAPs accompany a marked increase in the number of both alpha- and beta-tubulin isotypes (14), suggesting the existence of many classes of microtubules, each of which may perform a series of specialized tasks within neurons and glia.

Although much is known about the intracellular localization of certain MAPs and tubulin (2, 6, 9, 13, 21, 31, 35), nothing has been reported regarding the localization of the tau polypeptides in brain. Early studies using a polyclonal antibody to tau demonstrated that staining could be obtained in certain fibroblasts but not in a glioma cell line (12). Available evidence suggests that, in vitro, MAP2 and tau can compete for the same class of binding sites on the microtubule wall (23, 38). Therefore, the possibility exists that they may not coexist in the same intracellular compartment in situ. To investigate the possibility that tau and MAP2 are differentially compartmentalized we have isolated and characterized a monoclonal antibody that binds to all of the tau polypeptides in both bovine and rat brains. Using this antibody we have demonstrated biochemically (in bovine brain) and immunocytochemically (in rat brain) that tau is primarily localized in axons.

MATERIALS AND METHODS

Monoclonal Antibody Selection and Production: BALB/c mice were immunized with phosphocellulose-purified (51) bovine MAPs, which were denatured by boiling in 0.1% SDS. Immunization was performed over a period of 2 mo, with the initial injection performed in Freund's complete adjuvant and subsequent injections (at 2-wk intervals) carried out using Freund's incomplete adjuvant. Each injection contained at least 0.5 mg SDS-denatured bovine MAPs administered at both an interperitoneal and a subcutaneous site. 5 d before fusion, a final injection was administered to two animals. Immunized spleens were dissected and dissociated, and the splenocytes were fused with SP2/o myeloma cells by the procedure of Kohler and Milstein (25, 26) as modified (49). The resultant hybrid cells were plated in 12 96-well culture dishes on a thymocyte feeder layer in RPMI medium supplemented with 15% horse serum, and 24 h after plating this medium was exchanged with selection medium containing hypoxanthine, aminopterin, thymidine. Clone formation was observed within 7 d and most wells were confluent 14 d after fusion. Of the 1,156 wells plated, 1,155 were positive for hybrid formation. Antibody production was assayed by enzyme-linked immunosorbant assay (ELISA) (50) (see below, Quantitative ELISA) using phosphocellulose MAPs in the solid phase. Of the 1,155 wells assayed, >800 were positive for anti-MAP antibody production. 156 of the most intensely positive clones were picked and expanded into 1-ml cultures. Upon reaching confluence, the individual clones were frozen in complete medium that contained 25% horse serum and 10% dimethyl sulfoxide. Aliquots of spent medium from each clone were tested by ELISA for binding to purified bovine MAP2 or tau. Of the 156 clones frozen, 31 expressed antibody to MAP2 whereas only 6 produced antibody that recognized bovine tau. The antibody-containing media from these six putative tau clones were tested on immunoblots of whole rat brain. Only one line, Tau-1, was cross-reactive in rat brain, exhibiting binding to several polypeptides in the tau region of the gel. This line was thawed and subcloned twice before injection into a BALB/c mouse for the production of antibody-containing ascites fluid. Cells obtained from sterilely drained ascites fluid were harvested by centrifugation, cultured, and subcloned once more before freezing. 11 ml ascites fluid was harvested from one mouse and chromatographed on protein A-Sepharose, yielding nearly 30 mg of Tau-1 antibody at a final concentration of 0.96 mg/ml. Except for fluctuation analysis, this affinity-purified antibody was used in all of the biochemical experiments described below. Immunocytochemistry was performed using spent tissue culture medium harvested from stationary phase Tau-1-producing hybridomas.

The MAP2 and tubulin monoclonal antibodies used in this study were those described by Caceres et al. (9).

Protein Determination, SDS PAGE, and Immunoblotting Procedures: Protein determinations were performed by the method of Lowry et al. (29) as modified (1). SDS polyacrylamide slab gels (17 × 11.5 × 0.15 cm) were of the formulation of Laemmli (27). The separating gel consisted of a 32-ml, 5–12% linear acrylamide gradient. Electrophoresis was performed at 20-mA constant current until the tracking dye reached the bottom of the gel (~7 h). After electrophoresis, gels were either stained with Coomassie Brilliant Blue R or transferred to nitrocellulose for subsequent immunoblot analysis. Transfer of polypeptides to nitrocellulose was performed by the method of Towbin et al. (45) with the exception that 130 mA constant current for 6 h was used to effect transfer. Blotted nitrocellulose sheets were blocked by gentle agitation in 5% nonfat dry milk (weight/volume) in 50 mM Tris-HCl, pH 7.6, 0.2 M NaCl (Tris-saline) for 45 min at room temperature (22) before overnight incubation at 4°C in 20 μg/ml of Tau-1 antibody in Tris-saline. After primary antibody incubation, blots were washed in Tris-saline and blocked again for 15 min in 5% nonfat dry milk, Tris-saline, and then placed in the same solution containing a 1/5,000 dilution of peroxidase conjugated goat anti-mouse IgG (Hyclone, Provo, UT) for 2–3 h at 4°C. The blots were then washed again three or four times in Tris-saline and placed into substrate solution containing 0.0075% (vol/vol) H_2O_2, 0.04% (wt/vol) 3, 3′ diaminobenzidine tetrahydrochloride in 50 mM Tris, 10 mM imidazole-HCl, pH 7.6 (43). The reaction was stopped after sufficient color had developed (~2–3 min) by immersing the blot in deionized water. After air drying on filter paper, the blots were photographed using a dark blue filter.

Microtubule Protein Purification: Microtubules were purified by the assembly–disassembly procedure of Shelanski et al. (39). Bovine brain tissue was dissected from either the head of the caudate nucleus (gray matter) or internal capsule (white matter) and suspended at a ratio of 1 g tissue/ml of polymerization buffer containing 100 mM Pipes, pH 6.8, 1 mM $MgSO_4$, 2 mM EGTA, 0.1 mM GTP. After homogenization and centrifugation in the cold at 110,000 *g* for 1 h, the supernatant was removed and either stored as "crude" or "soluble" extracts or used for microtubule purification. Assembly, centrifugation, and cycling of microtubule protein were as described by Kim et al. (24). Tubulin was purified from twice-cycled microtubules using phosphocellulose chromatography (51). MAP2 and tau were purified by a modification of the method of Kim et al. (24) using heat precipitation and chromatography of the heat-stable fraction in high salt on a BioGel A-1.5m column (Bio-Rad Laboratories, Richmond, CA) (23).

Quantitative ELISA: The molar ratio of tau to tubulin was determined in microtubules assembled from both gray matter (caudate nucleus) and white matter (internal capsule). This was accomplished using a competitive ELISA. Purified tau or tubulin was attached to microtiter plates at a concentration of 0.01 mg/ml. The plates were washed and blocked in a solution containing 20 mM sodium phosphate buffer, pH 7.4, 0.13 M NaCl, 0.05% Tween-20, 0.4% BSA (bovine serum albumin) (PBS-TA). Initial serial antibody dilutions were performed to determine the antibody concentration that yielded an absorbance value corresponding to 60% of the maximal absorbance obtained. This antibody concentration was held constant in the presence of a serial dilution of unbound protein (either purified tubulin, tau, or an unknown sample). The antibody–protein suspension was preincubated at 37°C for 1 h and then plated onto the microtiter plates which contained preattached tubulin or tau (see above) and allowed to incubate for 1 h more at 37°C. After incubation, the plates were washed in PBS-TA and incubated in the presence of a second antibody peroxidase conjugate for 1 h at room temperature. The plates were again washed with PBS-TA, and an equal volume of substrate solution composed of pH 5.0 citrate–phosphate buffer, 0.25 mg/ml *o*-phenylenediamine, and 0.003% H_2O_2 was added to each microtiter well. After 20 min, the reaction was stopped with an equal volume of 0.65 M H_2SO_4, and the plates were read immediately on an ELISA reader at 492 nm. Standard curves were constructed by competing known amounts of tau or tubulin in the soluble phase with the appropriate antibody and measuring the amount of uncomplexed antibody able to bind to bound tau or tubulin. The amount of tau or tubulin in unknown samples was determined by extrapolation of the linear regions of the unknowns to the linear portions of the standards.

Immunohistochemical Procedures: Male Sprague-Dawley rats, 150–250 g, were anesthetized with pentobarbital (50 mg/kg) and intracardially perfused with 500 ml phosphate buffer (96 mM K_2PO_4, 24 mM NaH_2PO_4, pH 7.4) containing 20 μM $CaCl_2$, 2% paraformaldehyde, 0.25% glutaraldehyde, and 15% (vol/vol) of a saturated picric acid solution (20). The temperature of the perfusate was adjusted to 38°C immediately before perfusion. After perfusion, the animal was decapitated and the head was immersed in fixative for 3–4 h. At this time, the brain was removed and stored in 4% paraformaldehyde in phosphate buffer at 4°C for 24 h. The next day, blocks of tissue were transferred to 50 mM Tris-HCl, 130 mM NaCl, 5 mM KCl, 5 mM $MgSO_4$, 1 mM EGTA, 0.05% NaN_3, pH 7.6, (TBS) at 25°C containing 100 mM DL-lysine and stored at 4°C with continuous agitation for 12–18 h. All procedures described below were performed in TBS solution unless otherwise specified.

The tissue blocks were embedded in 5% molten agar, sectioned to a thickness ranging from 40 to 60 μm with a vibratome, and the resultant sections were placed into ice-cold TBS and stored at 4°C for no more than 24 h.

The unlabeled antibody method (42) was used to identify the distribution of anti-beta-tubulin, anti-MAP2, and anti-tau binding sites. Free-floating sections were blocked for 3 h at 30°C in TBS containing 5% BSA and 1% normal rabbit serum before being placed in various dilutions of primary antibody in TBS containing 0.5% BSA and 0.1% normal rabbit serum. Upon removal from primary antibody, sections were washed extensively in TBS without azide before unlabeled antibody treatment as described by Sternberger (42). After removal from peroxidase-anti-peroxidase complex, the sections were again exhaustively washed in TBS without azide and then immersed in chromogen solution containing 50 mM Tris-HCl, pH 7.6, at 25°C, 10 mM imidazole, 0.04% 3,3′ diaminobenzidine tetrahydrochloride, and 0.0075% H_2O_2 (43). The reaction was stopped by immersing the sections in ice-cold TBS. After more washes, the sections were mounted on gelatinized slides, air-dried overnight and dehydrated in ethanol, cleared in xylene, and mounted.

RESULTS

Analysis and Specificity of Tau-1

The monoclonal antibody secreted by the hybridoma line Tau-1 was shown by double immunodiffusion tests using isotype specific rabbit anti-mouse IgG antibodies to be of the IgG2a subclass (data not shown). As expected, Tau-1 binds to the four polypeptides that compose purified bovine tau (Fig. 1 *E*). Like a polyclonal antiserum recently reported (15), Tau-1 binds to five polypeptides in extracts of gray and white matter (Fig. 1, *A* and *B*). The binding of a single monoclonal antibody to all five tau species in bovine brain is not unexpected since the different tau polypeptides are known to have very similar peptide maps (11). Nevertheless, the binding of Tau-1 to five polypeptides in bovine brain raises the possibility that the hybridoma line producing the Tau-1 antibody is impure and is therefore producing a mixture of tau antibodies. To eliminate this possibility, we subcloned our Tau-1 producing hybridoma line an additional time and picked seven independent clones. The antibody expressed by each individual clone was tested against adjacent strips of blotted, purified tau. All clones produced antibodies that bound to the same four bands as the affinity purified Tau-1 antibody (Fig. 1 *E*), thus proving that Tau-1 is a monoclonal antibody.

Biochemical Localization of Tau

MAP2 and tau can bind to the same class of sites on the microtubule polymer in vitro (23, 38). However, immunocytochemical studies demonstrate that MAP2 is localized mainly in the dendrites of neurons in situ. Furthermore, analysis of microtubules purified from white matter– and gray matter–enriched areas of brain also indicates a paucity of MAP2 in in vitro–assembled microtubules from axon enriched regions (48). Since MAP2 is known to bind more tightly to in vitro–assembled microtubules than is tau (23, 38), we hypothesized that tau would be differentially compartmentalized, perhaps being more abundant in the axonal or glial portions of the nervous system. To test this, the soluble extracts and the twice-cycled microtubules from dissected white matter of the internal capsule were compared by immunoblotting with those from the head of the caudate nucleus, a highly enriched gray matter brain region. Equal loadings of protein from soluble, crude extracts prepared from gray and white matter demonstrate that significantly more tau is present in white matter preparations (compare Fig. 1, *A*, lanes *1* and *2* with *B*, lanes *1* and *2*). Similarly, more tau immunoreactivity is evident in white matter twice-cycled microtubules than in equal loadings of microtubules assembled from gray matter extracts (compare Fig. *1*, *D*, lanes *1* and *2* with *C*, lanes *1* and *2*). In addition, although five immunoreactive tau species are present in soluble extracts from both white and gray matter (Fig. 1, *a* and *b*), during microtubule purification from gray matter extracts, the lowest molecular weight tau species (tau*) apparently binds poorly to the microtubule polymer and is efficiently removed after two cycles of assembly (Fig. 1 *C*). In contrast, all five tau species in white matter extracts (Fig. 1 *B*) appear to co-purify with microtubules through two cycles of assembly (Fig. 1 *D*).

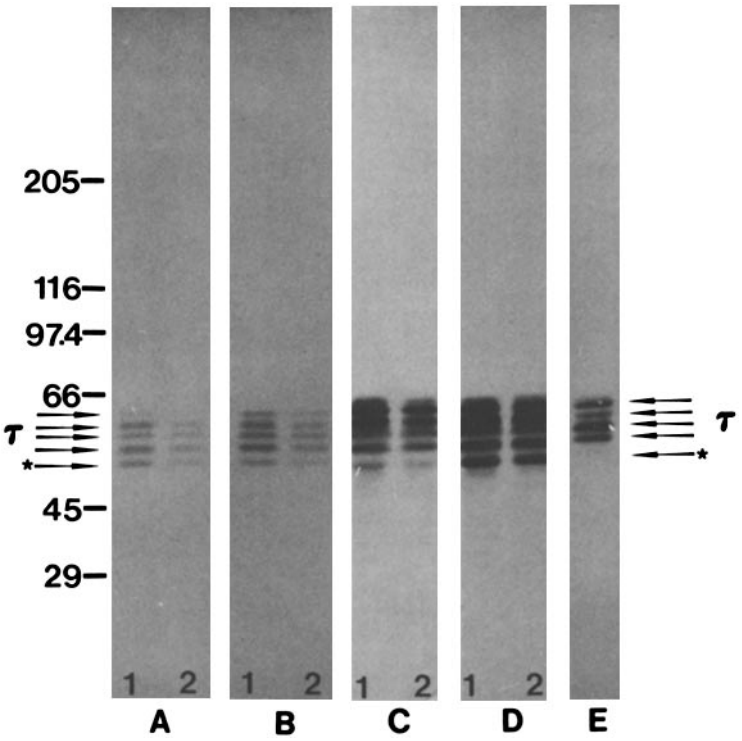

FIGURE 1 Qualitative examination of the relative amounts of tau in gray matter vs. white matter extracts and twice-cycled microtubules at two protein concentrations. Immunoblots of (*A*) a soluble extract from gray matter and (*B*) a soluble extract from white matter. The amount of protein loaded in *A*, lane *1* equals the amount loaded in *B*, lane *1*, and the amount of protein loaded in *A*, lane *2* equals the amount loaded in *B*, lane *2*. Immunoblots of twice-cycled microtubule preparation from (*C*) the gray matter extract shown in *A*, and (*D*) the white matter extract shown in *B*. The same amounts of protein were loaded in *C*, lane *1* and *D*, lane *1*. The amount of protein loaded in *C*, lane *2* equals the amount of protein loaded in *D*, lane *2*. (*E*) 25 ng of a purified bovine tau standard. Note the enrichment of tau* in *D* as compared with *C*, and also note that there is qualitatively more tau in both white matter extracts and microtubules than in corresponding preparations from gray matter. The numbers to the left represent molecular weight ($\times 10^{-3}$).

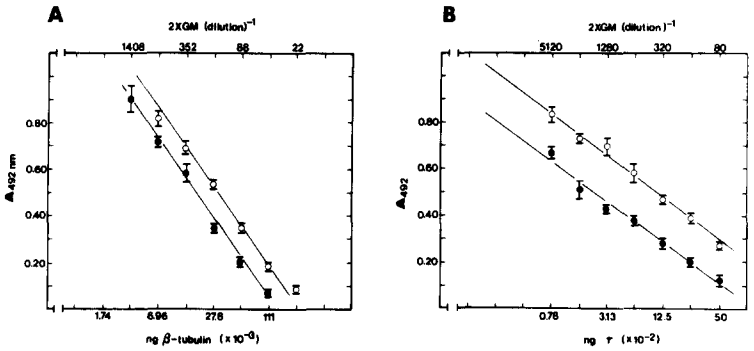

FIGURE 2 Quantitative determination of the amount of (*A*) tubulin and (*B*) tau in twice-cycled microtubules from a gray matter extract by competitive ELISA. In both assays the filled circles denote the standard curve and the open circles represent data from the unknown microtubule preparation *2XGM*, twice-cycled gray matter microtubules. Each point is an average of six determinations ±1 SD. (*A*) ●, β-tubulin. O, 2XGM. (*B*) ●, τ. O, 2XGM.

To quantify the difference between the microtubules purified from white and gray matter–enriched brain regions (Fig. 1, *C* and *D*) competitive ELISAs were performed (see Materials and Methods). We determined tau-to-tubulin ratios in the unknown samples of whole microtubules by the extrapolation to standard curves constructed using constant amounts of purified tau or tubulin bound to the microtiter plates. Serial dilutions of unbound tau or tubulin that had been preincubated with constant amounts of either Tau-1 or an anti-beta-tubulin monoclonal antibody were then added to the ELISA plates, and the relative amounts of bound antibody were measured. An example of such a determination is shown in Fig. 2, *A* and *B*, and the final results are summarized in Table I. Whereas the tau-to-tubulin ratio in gray matter microtubules is 1 tau per 38.4 tubulin dimers, the ratio in white matter microtubules is three times higher at 1 tau per 12.8 dimers. When combined with the data shown Fig. 1, *C* and *D* these results indicate that both qualitatively and quantitatively, more tau is present in white matter microtubules than in those purified from gray matter. Moreover, since gray matter preparations can contain as much as 40% axons by volume (8) in addition to neuronal cell bodies and dendrites, while white matter preparations contain virtually no neuronal cell bodies or dendrites, tau would appear to be predominately axonal. However, both preparations also contain a multitude of glial cells. For this reason, immunohistochemical studies were performed to determine more precisely the localization of tau in brain tissue.

TABLE I. *The Tau-to-Tubulin Ratios Obtained from Competitive ELISAs Performed on Twice-cycled Microtubules from Both Gray and White Matter Extracts*

	White matter MT	Gray matter MT
τ (*mg*)	0.19 ± 0.01	0.08 ± 0.01
β-Tubulin (*mg*)	2.19 ± 0.19	2.77 ± 0.10
Molar ratio (*τ/tubulin*)	1/12.8	1/38.5

The indicated amounts of protein (in milligrams) have been standardized to 1 ml. The data are expressed as an average ± 1 SD. The molar ratios were calculated using molecular weights of 55,000 for beta-tubulin and 61,000 for tau. MT, microtubules.

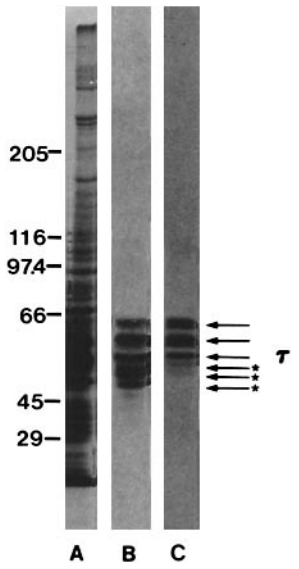

FIGURE 3 Specificity of Tau-1 in whole rat brain. Lane *A*, Coomassie Blue stain of an SDS extract of whole rat brain. Lane *B*, immunoblot of a lower concentration of the sample in lane *A* reacted with the Tau-1 monoclonal antibody. Unlike bovine brain, rat brain contains at least six immunoreactive species of tau. Lane *C*, immunoblot of a twice-cycled microtubule preparation from whole rat brain. Note that two or three polypeptides (*) are lost during microtubule purification. The numbers to the left represent molecular weight (×10^{-3}).

Immunohistochemical Localization of Tau in Brain

Immunohistochemical studies were performed on adult rats after aldehyde fixation by vascular perfusions. Before we did this, we first had to establish the fidelity of our antibodies in binding to tau in rat brain. Immunoblot analysis of whole rat brain SDS extracts indicated that at least six electrophoretic species were present in the tau region of the gel (Fig. 3, lane *B*). As in standard microtubule preparations from bovine brain (15), not all of these polypeptides co-purify with tubulin during temperature-dependent cycles of assembly and disassembly (Fig. 3, lane *C*). During microtubule purification from rat brain, it appears that two or three polypeptides (also designated as tau*) are lost. Furthermore, two of the three remaining major tau species resolve as doublets on our gel system (Fig. 3, lane *C*), indicating an enrichment of minor tau polypeptides during microtubule protein purification from rat brain.

Using the unlabeled antibody technique (42), we examined the distribution of tau immunostaining in rat brain at the level of the light microscope. After fixation (see Materials and Methods) vibratome sections were cut, and tissue staining was performed in cerebellum, medulla, and substantia nigra. In each case, tau immunostaining was contrasted with staining by both anti-beta-tubulin and anti-MAP2 monoclonal antibodies. In the hypoglossal nucleus of the medulla, tubulin staining was seen in cell bodies, dendrites, and axons (Fig. 4 *A*) whereas MAP2 was present mainly in dendrites and cell bodies, with little axonal staining observable (Fig. 4*B*). In contrast, tau localization was confined mainly to axons with no staining detectable in either cell bodies or dendrites (Fig. 4*C*). In the substantia nigra, cell bodies, dendrites, fine axonal fibers, and the axonal fiber bundles of the cerebral peduncles stained with anti-tubulin antibody (Fig. 5*A*). Again, MAP2 localized most intensely in the neuronal cell bodies and dendrites (Fig. 5*B*), and tau localization was largely comple-

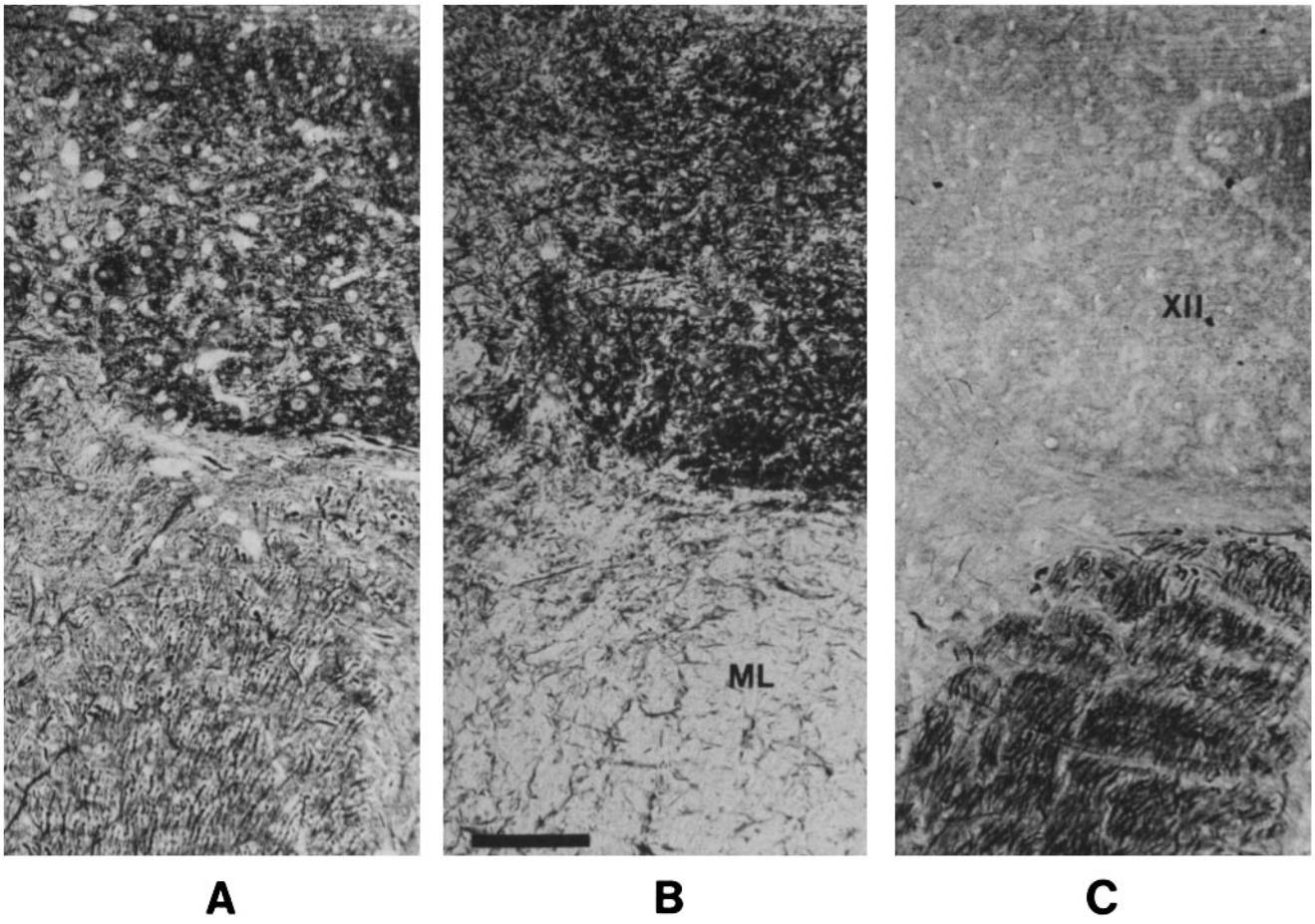

FIGURE 4 Low-power micrographs of vibratome sections through the hypoglossal nucleus in the medulla immunostained with (*A*) an anti-beta-tubulin monoclonal antibody, (*B*) an anti-MAP2-monoclonal antibody, and (C) Tau-1. *ML*, medial lemniscus, a large axon bundle. *XII*, the cell mass of the hypoglossal that gives rise to the twelfth cranial nerve. Note that the cell bodies and dendrites are stained by anti-tubulin and -MAP2 antibodies but not by Tau-1. The axons of the medial lemniscus are stained by anti-tubulin and Tau-1 but not by an anti-MAP2 monoclonal antibody. Bar, 80 μm.

mentary to the anti-MAP2 staining in that all observable anti-tau immunostaining appeared in the fine axonal fibers of the substantia nigra and in the peduncles (Fig. 5 *C*). Again, no tau localization was seen in cell bodies.

We have also examined tau localization in the cerebellum. The most prominent immunoreactive processes in the molecular layer are the small parallel fiber axons (Fig. 6, *A* and *B*). As in the other brain areas examined the dendrites and glia were unstained by Tau-1. However, the result in the cerebellum is somewhat more striking since the heavy staining of the parallel fiber axons causes the unstained Purkinje cell bodies and dendrites as well as the processes of Bergmann glia to appear in negative relief (Fig. 6, *A* and *B*).

DISCUSSION

Biochemical experiments demonstrate that the concentration of tau in bovine brain tissue is greater in soluble extracts of white matter than in gray matter. In addition, microtubules made from white matter contain about threefold more tau than those purified from gray matter, and although tau is electrophoretically hetergeneous, all of its observable forms are found in white matter preparations. Although five tau polypeptides are observed in extracts of bovine white and gray matter–enriched preparations, only four co-purify with gray matter microtubules whereas all five co-purify with white matter microtubules. The polypeptide species that is lost during microtubule purification (tau*) was previously shown by Cleveland et al. (11) to be present in tau purified directly from crude extracts of brain tissue by heat treatment but was absent or present in greatly reduced quantities in microtubules purified by temperature-dependent cycles of assembly-disassembly (15).

Immunocytochemical studies in rat brain using a monoclonal antibody that recognizes an epitope common to all of the tau polypeptides in bovine and rat brain extracts and microtubule preparations extend these observations by revealing that tau immunoreactivity is restricted to the axonal compartment of neurons and does not localize in glia. The exclusive localization of tau in axons as seen with our methods is supported by the following biochemical determinations. First, data from quantitative ELISAs show that there is three times more tau in microtubules made from white matter than in those assembled from gray matter. Because gray matter contains axons as well as dendrites and cell bodies (measurements indicate that a conservative estimate for axonal volume in gray matter is ~40% [8]), it is clear that some of the tau protein of gray matter arises from axonal sources. Second, since our immunoblots indicate that there is less tau in soluble extracts of gray matter than in white matter, the other cellular

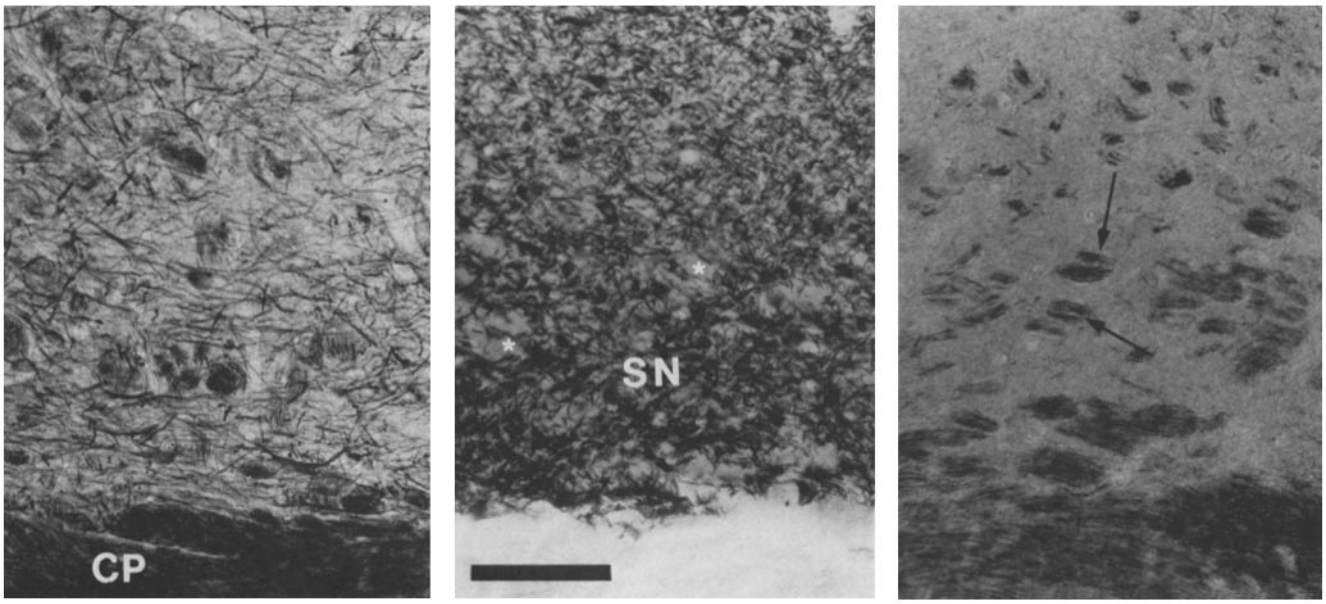

FIGURE 5 Low-power micrographs of vibratome sections through the substantia nigra (*SN*) immunostained with (*A*) anti-beta-tubulin, (*B*) anti-MAP2, and (*C*) Tau-1. Note that the cerebral peduncles (*CP*), an axon bundle, are stained intensely by the anti-beta-tubulin antibody (*A*) and Tau-1 (*C*), whereas only light staining is observable upon staining with an anti-MAP2 antibody (*B*). Anti-tubulin and -MAP2 antibodies stain the dendrites and cell bodies (*A* and *B*); these are unstained by Tau-1 (*C*). The arrows point to fiber bundles in the substantia nigra that are stained with Tau-1 (*C*); a few of the corresponding areas not stained by anti-MAP2 (*B*) are indicated with asterisks. Bar, 80 μm.

and subcellular compartments probably contain little tau. Such independent biochemical data support our immunocytochemical observations but do not prove that tau is exclusively axonal. Epitope masking due to protein–protein interactions or conformational changes in the tau molecule may result in false negative determinations in other cellular and subcellular compartments. These caveats noted, however, it is clear from the data presented that most tau is axonal. In support of this, sympathetic neurons in culture have been subjected to the same biochemical analyses. In these cells, it is possible to separate physically the axonal compartments from the cell body–dendritic compartments. Analysis of both preparations using either Tau-1 or a polyclonal antibody to tau also demonstrates that tau is largely axonal (Peng, I., L. I. Binder, and M. M. Black, manuscript in preparation). The mechanisms underlying such differential localization are not known. We will discuss two hypotheses. The first involves differential MAP binding by different tubulin isoforms, and the second involves regional synthesis of MAP2 and tau.

Competition studies comparing the relative affinities of MAP2 and tau for intact microtubules in vitro have shown that two classes of binding sites exist on in vitro-assembled microtubules (23, 38). Although both classes allow binding of either protein, MAP2 will displace tau from the MAP2 site and tau will displace MAP2 from the tau site. Most of these sites have a high affinity for MAP2 and the rest display a higher affinity for tau than MAP2. It is pertinent, however, that most standard tubulin preparations are made from extracts of bovine cerebral cortex, an enriched gray matter region of the brain containing a composite of dendrites, unmyelinated axons, neuronal cell bodies, and glia; it is not known whether similar results would be obtained if tubulin isolated from white matter were used in these experiments. That is, it is not known whether tau binds more tightly than MAP2 to white matter (axonal) tubulin. Indeed, the fact that tau* cycles with white matter tubulin but not gray matter tubulin strongly suggests that the tubulins of these two regions differ, perhaps in isoform composition. Supporting this, differential localization of different tubulins within single cells in culture has been demonstrated (19, 44). Thus, our hypothesis suggests that the different MAP binding domains found on in vitro–assembled microtubules from gray matter are specified by compartment-specific tubulins. MAP2-tau competition experiments performed on microtubules isolated from both gray matter and white matter regions of the brain provide a means of testing this hypothesis. A second hypothesis, which to us seems less likely, is that differential localization of tau and MAP2 arises not because the underlying tubulins in cell bodies and dendrites are different from those in axons but because MAP2 and tau are synthesized in different regions of the neuronal cell body. Although MAP2 is known to be present in cell bodies (9, 13, 17), we have been unable to detect tau in the cell body proper (see below). Perhaps tau is synthesized at or near the initial segment of the axon and MAP2 is synthesized in a different cell body compartment.

Indeed, a perplexing aspect of the localization of Tau-1 is the fact that the neuronal cell bodies are unstained. The reason for this is unclear. Since axons contain no protein synthesizing machinery, tau must be synthesized in the cell bodies and transported into the axon. Either tau is in a form in cell bodies that will not bind the antibody or it is synthesized very near the somal–axonal junction and shunted immediately into the axons. This hypothesis could be tested by in situ hybridization studies using labeled tau cDNA to localize tau mRNA.

The work presented here provides few clues about the

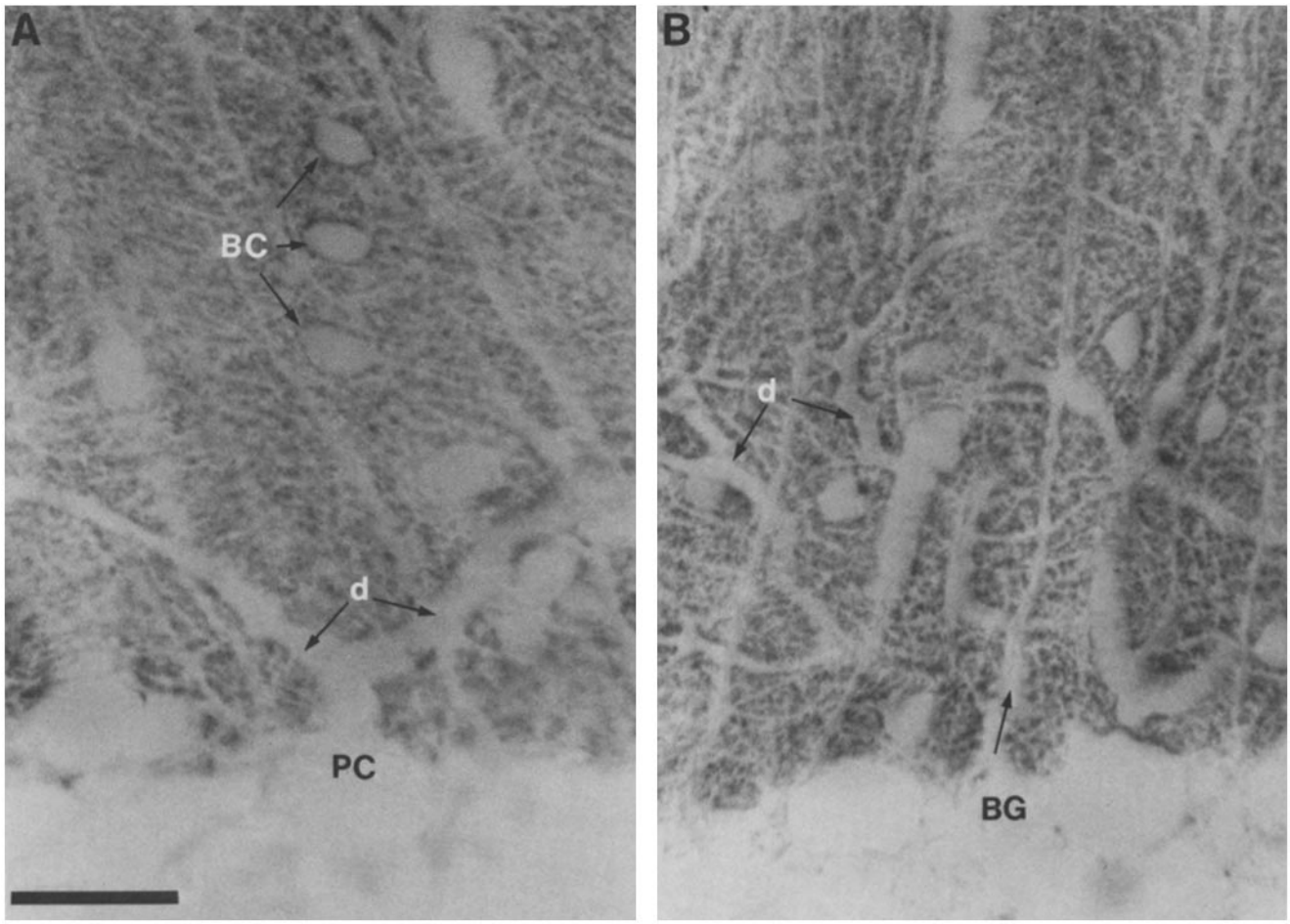

FIGURE 6 High-power micrographs of vibratome sections through a portion of the Purkinje cell and molecular layers of the cerebellum immunostained with Tau-1. (*A*) Section through an unstained Purkinje cell body (*PC*) showing continuity with its unstained dendrites (*d*) that extend into the molecular layer towards the pial surface. Also unstained are the basket cell bodies (*BC*). (*B*) Section showing unstained processes of Bergmann glia (*BG* plus arrow), as well as numerous unstained Purkinje cell dendrites (*d*). The stippled appearance provided by the positive staining in the molecular layer is due to the presence of thousands of parallel fiber axons that contain abundant tau. Bar, 25 μm.

functional reasons for the heterogeneity of tau. The Tau-1 monoclonal antibody reacts with all of the known tau polypeptides in both rat and bovine brain. In bovine brain, extracts of the internal capsule contain all of the known tau species. However, additional experiments have shown that different tau species are predominant in different brain regions (Binder, L. I., A. Frankfurter, and L. I. Rebhun, manuscript in preparation, and reference 37). Using a polyclonal antibody that reacts to all of the known tau polypeptides, Drubin et al. (15) have reported that the major immunoreactive species in PC12 cells is an M_r 110,000–130,000 species. We have found that the major Tau-1 immunoreactive species in optic and peripheral nerve (36, 37) is a doublet or triplet of polypeptides at M_r 105,000–130,000 that accompanies varying numbers of the lower molecular weight tau bands (data not shown). In particular, it is interesting that in addition to the high-molecular weight tau bands in optic nerve, we find predominantly two of the mid-molecular weight tau polypeptides, in agreement with the report of Tytell et al. (47). It is highly unlikely that this heterogeneity is without function since during postnatal development the number of tau polypeptides changes from a single species early in development to the more complex adult forms by postnatal day 20 (16, 18). These changes parallel alterations in the electrophoretic complexity of MAP2 (4, 7) and tubulin (14), which suggests that they are all tied to a common neuronal maturation process. Since our data suggest that tau is restricted to axons, developmental increases in the electrophoretic complexity of tau imply changes in the biochemical composition of axonal microtubules as compared with the microtubules in neuronal cell bodies, dendrites, and glia.

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