Synthesis, Axonal Transport, and Turnover of the High Molecular Weight Microtubule-associated Protein MAP 1A in Mouse Retinal Ganglion Cells: Tubulin and MAP 1A Display Distinct Transport Kinetics

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Abstract. Microtubule-associated proteins (MAPs) in neurons establish functional associations with microtubules, sometimes at considerable distances from their site of synthesis. In this study we identified MAP 1A in mouse retinal ganglion cells and characterized for the first time its in vivo dynamics in relation to axonally transported tubulin. A soluble 340-kD polypeptide was strongly radiolabeled in ganglion cells after intravitreal injection of [³⁵S]methionine or [³H]proline. This polypeptide was identified as MAP 1A on the basis of its co-migration on SDS gels with MAP 1A from brain microtubules; its co-assembly with microtubules in the presence of taxol or during cycles of assembly–disassembly; and its cross-reaction with well-characterized antibodies against MAP 1A in immunoblotting and immunoprecipitation assays. Glial cells of the optic nerve synthesized considerably less MAP 1A than neurons. The axoplasmic transport of MAP 1A differed from that of tubulin. Using two separate methods, we observed that MAP 1A advanced along optic axons at a rate of 1.0–1.2 mm/d, a rate typical of the Group IV (SCb) phase of transport, while tubulin moved 0.1–0.2 mm/d, a group V (SCa) transport rate. At least 13% of the newly synthesized MAP 1A entering optic axons was incorporated uniformly along axons into stationary axonal structures. The half-residence time of stationary MAP 1A in axons (55–60 d) was 4.6 times longer than that of MAP 1A moving in Group IV, indicating that at least 44% of the total MAP 1A in axons is stationary. These results demonstrate that cytoskeletal proteins that become functionally associated with each other in axons may be delivered to these sites at different transport rates. Stable associations between axonal constituents moving at different velocities could develop when these elements leave the transport vector and incorporate into the stationary cytoskeleton.

MICROTUBULES are composed of tubulin and a diverse group of microtubule-associated proteins (MAPs)[1] that are defined by their persistent association with microtubules after repeated cycles of tubulin assembly and disassembly in vitro (Murphy and Borisy, 1975; Sloboda et al., 1976). In the nervous system, certain MAPs seem to impart distinctive functional characteristics to the microtubule populations in different neuronal compartments (Hirokawa et al., 1988). MAP 2 comprises a group of three closely related phosphoproteins, MAP 2A and MAP 2B (both at ~280 kD), and a 70-kD polypeptide, MAP 2C, which are abundant in dendrites and rare in axons (Matus et al., 1981; Vallee, 1980; Caceres et al., 1984; DeCamilli et al., 1984). Tau proteins (45–60 kD), another fam-

ily of phosphoproteins, are enriched in axons (Weingarten et al., 1975; Cleveland et al., 1977; Lindwall et al., 1984; Binder et al., 1985). MAP 1 polypeptides (~340 kD) are less well-characterized. Although nomenclatures for individual MAP 1 forms differ among laboratories, at least three polypeptides are distinguished on the basis of structural, developmental and immunocytochemical studies (Bloom et al., 1984; Schoenfeld et al., 1989; for review, Vallee, 1984; Olmsted, 1986; Matus, 1988). MAP 1A, or MAP 1, distributes throughout axons and dendritic processes and is expressed at increasing levels as development progresses (Bloom et al., 1984; Herrmann et al., 1985; Huber and Matus, 1984; Herrmann et al., 1985; Luca et al., 1986). MAP 1B and its putative cellular isotypes, MAP 5 (Riederer et al., 1986), MAP 1X (Calvert and Anderton, 1985), and MAP 1.2 (Asai et al., 1985), are abundant early in development

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1. *Abbreviation used in this paper:* MAP, microtubule-associated protein.

and decrease markedly in adulthood (Bloom et al., 1985; Calvert and Anderton, 1985; Riederer et al., 1986; Safaei and Fischer, 1989). MAP 1A and MAP 1B appear to have little structural similarity (Bloom et al., 1985; Herrmann et al., 1985); however, both proteins associate with similar low molecular weight polypeptides (Vallee and Davis, 1983; Schoenfeld et al., 1989). MAP 1C, a dynein-like microtubule-activated ATPase (Paschal and Vallee, 1987), is structurally distinct from other high molecular weight MAPS.

Various roles have been ascribed to MAPs in regulating the assembly, stability, and spatial organization of microtubules. Tau promotes the assembly of microtubules (Weingarten et al., 1975) and, like MAP 2, stabilizes these structures partly by inhibiting their disassembly (Black and Greene, 1982; Drubin and Kirschner, 1986; Kosik et al., 1988; Schliwa et al., 1981). MAPs may also regulate interactions of microtubules with each other and with other cytoskeletal proteins (Olmsted, 1986). MAP 2 projects radially from the microtubule surface (Vallee, 1980) and, in dendrites, forms a crossbridge between microtubules and neurofilaments (Leterrier et al., 1982; Heimann et al., 1985; Hirokawa et al., 1988). MAP 1A may compose cross-bridges between microtubules (Hirokawa, 1982; Shiomura and Hirokawa, 1987a,b). The appearance of MAP 1A in neurons relatively late in postnatal development coincides with changes in the organization of the cytoskeleton that may stabilize a particular neuronal shape or intracellular latticework. Although electron microscope images and in vitro assembly assays seem to confirm the physical association between MAPs and microtubules, relatively little is known about the dynamics of these interactions in vivo.

One potential strategy for investigating the dynamic properties of MAPs is to examine their behavior in relation to other cytoskeleton-associated proteins as they are translocated along axons after synthesis. The major proteins of the axonal cytoskeleton are translocated as constituents of the two slowest moving phases of axoplasmic transport, designated Group IV or slow component b (SCb), and Group V or slow component a (SCa) (Hoffman and Lasek, 1975; Willard and Hulebak, 1977; Grafstein and Forman, 1980). Group IV, which advances at a rate of 2–20 mm/d, is composed of >100 different proteins, including actin, fodrin, myosin-like protein, clathrin, and many metabolic enzymes (Black and Lasek, 1979; Willard et al., 1979; Willard, 1977; Garner and Lasek, 1981; Brady and Lasek, 1981). By contrast, Group V, which advances at a rate of 0.1–1.0 mm/d, is predominantly composed of neurofilament proteins and tubulin, as well as smaller amounts of fodrin and actin (Hoffman and Lasek, 1975; Black and Lasek, 1980). Although neurofilament proteins are transported principally as polymers (Nixon and Lewis, 1986; Hoffman and Lasek, 1975), tubulin transport is more complex (see reviews, Hollenbeck, 1989; Nixon, 1990). Growing evidence indicates that some tubulin moves as soluble dimers (Letourneau and Ressler, 1984; Okabe and Hirokawa, 1988; Tashiro and Komiya, 1989; Lim et al., 1989; Bamburg et al., 1986) which then insert into the distal (+) ends of preexisting microtubules (Bamburg et al., 1986; Okabe and Hirokawa, 1988). Another fraction of tubulin may move in association with insoluble structures representing either assembled microtubules (Black et al., 1986; Tashiro and Komiya, 1989) or aggregates of unassembled subunits or protomers (Hollenbeck and Bray, 1987; Weisenberg et al., 1988).

Several subtypes of tau protein are transported at a Group V rate (Tytell et al., 1984), which has suggested that these polypeptides are attached to microtubules undergoing transport. The possibility has not been excluded, however, that tau protein is transported in association with an unassembled insoluble structure containing Group V components (Weisenberg et al., 1987; Hollenbeck and Bray, 1987) or with neurofilament proteins, which can bind tau in vitro (Miyata et al., 1986). Aside from tau proteins, no information is available about the axoplasmic transport of other MAPs.

In this study, we characterized the synthesis, axonal transport, and subsequent fate of a 340-kD MAP. Using biochemical, immunochemical, and functional criteria, we have identified this polypeptide as MAP 1A and provided the first information about its in vivo dynamics. Although MAP 1A and microtubules appear to form functional associations within axons in vivo, we observed that MAP 1A and tubulin are transported at different rates. A further observation, that some transported MAP 1A incorporates into a slowly-turning-over stationary cytoskeleton, together with recent evidence for populations of stationary microtubules (Lim et al., 1989; Okabe and Hirokawa, 1988), explains how constituents of two different transport groups might become physically associated in vivo. These findings support the concept that a major portion of the axonal cytoskeleton is assembled within axons. The localization in axons of mechanisms for assembling the cytoskeleton implies a capability for local reorganization of the cytoskeleton in response to physiological stimuli and for regional specializations of the cytoskeleton along axons and within synapses.

Materials and Methods

Isotope Injections

Radiolabeled amino acids were injected intravitreally into anesthetized male and female C57Bl/6J mice, aged 10–14 wk, with a calibrated micropipette apparatus (Nixon, 1980). Mice received 0.25 μl of PBS, pH 7.4, which contained 25 μCi of L-[2,3-^{3}H]proline (specific activity 30–50 Ci/mmol) or 50–100 μCi of L-[^{35}S]methionine (specific activity 400 Ci/mmol) purchased from New England Nuclear (Boston, MA).

The following additional measures were taken in studies on the kinetics of MAP disappearance to reduce variation in amounts of radiolabeled amino acid incorporated into axonal proteins in different animals. For a single experiment involving 80–100 mice, identical volumes of labeled precursor solution were injected into all animals within a single 4-h period using calibrated micropipettes. Repeated experiments were carried out at the same time of day to avoid possible circadian variation in protein synthesis or axonal transport. To reduce the risk of leakage from the injection site, the bore of the micropipette (70 μm) and the site and depth of the injection were standardized. A given animal was not analyzed as part of the series if leakage of the solution out of the eye was observed (Nixon and Logvinenko, 1986).

Tissue Preparations

Mice were killed by cervical dislocation. After the brain was removed and cooled, the optic nerve and optic tract were freed from meninges and the optic tract on each side was severed at a point 2.5 mm from the superior colliculus. This dissected length of optic pathway, referred to as optic axons, was 9 mm long and consisted of the optic nerves severed at the scleral surface of the eye, the optic chiasm, and a length of the optic tract extending to, but not including, terminals in the lateral geniculate nucleus. In experiments involving the radiolabeling of glial cell proteins in vitro, the optic pathway was cut at the midpoint of the optic chiasm to obtain an optic nerve sample for analysis. In other experiments, the optic pathway was cut into consecutive 1.1-mm segments on a micrometer slide. All manipulations were performed at 4°C.

Radiolabeling of Glial Cell Proteins of the Optic-Pathway In Situ

Intact, unlabeled optic pathways, freshly dissected from mice, were incubated for 60 min at 37°C in 0.5 ml of a Hepes medium (25 mM Hepes, 5 mM KCl, 110 mM NaCl, 50 mM glucose, pH 7.4) containing 75 μCi of [^{35}S]methionine (specific activity 400 Ci/mmol). The medium was then replaced by 1.0 ml of the same medium without radioisotopic amino acid but containing protein synthesis inhibitors (0.5 mM cycloheximide) (Sigma Chemical Co., St. Louis, MO) and 0.3 mg/ml chloramphenicol (Sigma Chemical Co.). Incubation in this medium was continued at 37°C for an additional 5 min. This was repeated once to remove unincorporated radioactivity, and the tissue was then frozen at −70°C until analyzed (Nixon, 1982).

Cytoskeleton and Microtubule Preparations

Cytoskeletal proteins and Triton X-100–soluble protein fractions were prepared from optic axons by the method of Chiu and Norton (1982). To minimize proteolysis, buffers in all experiments contained 50 μg/ml leupeptin, 0.5 mM PMSF, and 2.5 mg/ml aprotinin. Microtubule fractions of optic axons and whole mouse brain were prepared from mouse brain by two cycles of in vitro assembly and disassembly in MT buffer (50 mM Pipes, pH 6.6, 1 mM MgSO$_4$, 1 mM EGTA) by the method of Shelanski et al. (1973). For co-assembly experiments, optic nerve homogenates were prepared in MT buffer containing 1 mM GTP with added brain microtubules. The mixture was centrifuged at 100,000 g for 30 min at 4°C, and the supernatant was incubated at 37°C for 30 min to allow the microtubules to polymerize. The microtubules were then spun at 40,000 g for 30 min at 30°C.

The microtubule fraction from segments of at least 10 individual radiolabeled optic nerves was prepared using the taxol method (Vallee, 1982). The tissue was homogenized in 1.5 vol of MT buffer, including protease inhibitors, and centrifuged in the cold at 100,000 g for 30 min. The supernatant was made 1 mM GTP and 10 μM taxol, incubated at 37°C to polymerize the microtubules, and centrifuged at 40,000 g. The pellet containing the microtubule fraction and the supernatant containing the nonmicrotubule fraction were used for PAGE analysis as described in other sections.

PAGE

One-dimensional SDS-PAGE was carried out by the method of Laemmli (1970) using 320-mm slab gels containing 5–15% or 3–7% polyacrylamide gradients (Nixon et al., 1986). Two-dimensional polyacrylamide gels containing SDS were prepared with 8–18% polyacrylamide gradients as previously described (Brown et al., 1981).

Identification and Quantification of Radioactive Proteins

Proteins labeled with [^{3}H]proline were detected by fluorography (Marotta et al., 1979). Proteins labeled with [^{35}S]methionine were detected by autoradiography. In some experiments gels were stained with Coomassie blue and the appropriate proteins for analysis were identified on gels by comparison with migration of known proteins in cytoskeleton or microtubule preparations. The protein bands were cut out and the radioactivity in gel slices (disintegrations per minute) was determined as previously described (Nixon et al., 1986).

Immunoprecipitation of MAPs

Antibodies against the different forms of MAP 1 were the generous gifts of Dr. George Bloom (University of Texas Southwestern Medical Center) and Dr. Lester Binder (University of Alabama at Birmingham). They included monoclonal antibodies against MAP 1A clone 1A-1 (Bloom et al., 1985) and clone M1.4 (Brown and Binder, 1989); and antibodies against MAP 1B clone 1B-5 (Bloom et al., 1985) and clone MAP 1/2 (Safaei and Fischer, 1989). In addition, we used a polyclonal antibody produced against a cloned fusion protein of MAP 1B (Safaei and Fischer, 1989).

Immunoprecipitation of MAPs from fractions of radiolabeled optic axons was performed as previously described (Fischer et al., 1986). Briefly, homogenates of optic axons were diluted with 4 vol of buffer containing 50 mM Tris-HCl, pH 7.6, 190 mM NaCl, 6 mM EDTA, 1.25% Triton X-100, and protease inhibitors. The mixture was first cleared from nonspecific binding by incubation with 50 μl protein A-Sepharose gel (Pharmacia Fine Chemicals, Piscataway, NJ) and centrifugation at 11,000 g for 1 min. The sample was immunoprecipitated by incubation with the appropriate antibodies for 16 h at 4°C, followed by addition of protein A-Sepharose for 2 h at room temperature. When monoclonal antibodies were used, a protein A-Sepharose-antibody complex was first prepared by coupling 100 μl of the protein A-Sepharose gel with 50 μl of rabbit anti-mouse antibodies (Cappel Laboratories, Malvern, PA). After an extensive washing with 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 5 mM EDTA, and 0.5% Triton X-100, the immunoprecipitated proteins were eluted by boiling in 100 μl of electrophoresis sample buffer for 3 min.

Quantitation of Total MAPs

Total protein content in tissue samples was determined by the method of (Lowry et al., 1951). The total content of MAP 1A at different levels of the optic pathway was determined by measuring the quantitative binding of Coomassie blue dye to proteins (Bradford, 1976) using a laser densitometer. Readings were made on enlarged Mylar transparency prints made from photographs of the relevant regions of Coomassie-stained gels. Protein concentrations on gels were within a range that yielded a signal proportional to the protein content. Density readings were corrected for variations in the width of gel lanes by measuring the total area of the stained band using a digitizing board (Summagraphics Corp., Fairfield, CT) interfaced with a Bioquant software package. Background equivalent to the density of a region in each gel lane that contained no protein band was subtracted to compare scans from different gel lanes. Density readings from different gels were normalized to yield the same total density for each replicate experiment. Scans for replicate samples on different gels were then averaged together. Fluorographs of two-dimensional gels were digitized in a 1,024 × 1,024 pixel format and analyzed using the Visage 110 image system (BioImage, Ann Arbor, MI) and its software package. Spot quantitation was computed from the spot boundary pixels and the integrated intensity of all the pixels within those boundaries.

Immunoblot Analysis

Proteins separated by SDS-PAGE were electrophoretically transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH)) for 1 h at 0.5 Amp in a Polyblot apparatus (American Bionetics, Inc., Emeryville, CA) as described previously (Fischer et al., 1987). The nitrocellulose paper was incubated at room temperature with gentle agitation in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl (TBS) containing 5% nonfat dry milk (Carnation, Los Angeles, CA) to block nonspecific binding. The nitrocellulose was then reacted overnight at 4°C with the primary antibodies diluted in the same solution. After a thorough washing for 30 min with TBS, the nitrocellulose paper was incubated with horseradish peroxidase–conjugated antibodies (Cappel Laboratories), for 1 h at room temperature, washed, and stained with diaminobenzidine. For quantitative analysis, the secondary antibody was replaced by ^{125}I-protein A and the nitrocellulose paper was washed, dried, and exposed against film for autoradiography.

Results

Identification of MAP 1A in Retinal Ganglion Cell Axons

The proteins in retinal ganglion cells that compose the slow phases of axoplasmic transport, Group IV (1–2 mm/d) and Group V (0.2–0.5 mm/d), were radiolabeled by intravitreal injection of [^{35}S]methionine and analyzed by SDS-PAGE. By 6 d after injection, proteins in both transport groups are well-represented (Fig. 1). The major constituents of each group have been previously characterized (Nixon and Logvinenko, 1986; Brown et al., 1982; Nixon et al., 1982; Nixon, 1986). In this study we identified a 340-kD protein that was visible by Coomassie blue staining and partitioned exclusively into the Triton-soluble fraction (Fig. 1). A protein corresponding in molecular mass to the 340-kD protein was heavily radiolabeled in optic axons 3 d after [^{35}S]methionine injection (Fig. 1). About 95% of the radioactivity associated with this protein partitioned into the Triton-soluble fraction. A protein of similar apparent molecular mass was
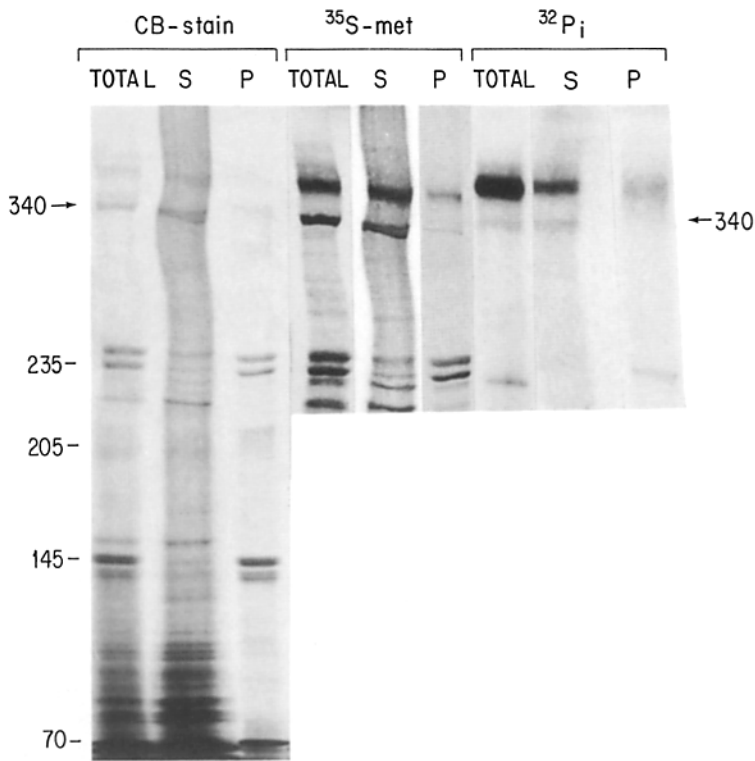
Figure 1. SDS-PAGE analysis of Coomassie blue–stained optic nerve proteins (*CB-stain*) and radiolabeled polypeptides in optic axons of mice 6 d after intravitreal injection of [^{35}S]methionine (^{35}S-met) or 3 d after intravitreal injection of ^{32}P-orthophosphate (^{32}P$_i$). Unfractionated optic axons (*TOTAL*) are compared with Triton-soluble fractions (*S*) and Triton-insoluble fractions (*P*). Each fraction is derived from one optic pathway (protein content: 152 μg total, 91 μg Triton-soluble, 61 μg Triton-insoluble). Total protein content was revealed by staining with Coomassie blue, and radiolabeled proteins were detected by autoradiography. The gels contained polyacrylamide gradients of 3–7%. The Coomassie blue and ^{35}S-labeled protein patterns are from the same three gel lanes. The 340-kD polypeptide is indicated by an arrow and molecular mass estimations from appropriate standards are provided.

labeled after intravitreal injection of ^{32}P-orthophosphate (Fig. 1).

To examine the relative expression of this protein in neurons and glial cells, we labeled glial proteins by incubating optic nerves in vitro with [^{35}S]methionine for brief periods under conditions that largely preserved the structural integrity of the axon and surrounding cells (Nixon, 1983; Nixon, 1986). This group of optic nerves and another group pooled from mice 6 d after intravitreal injection of [^{35}S]methionine were each partitioned into Triton-soluble and
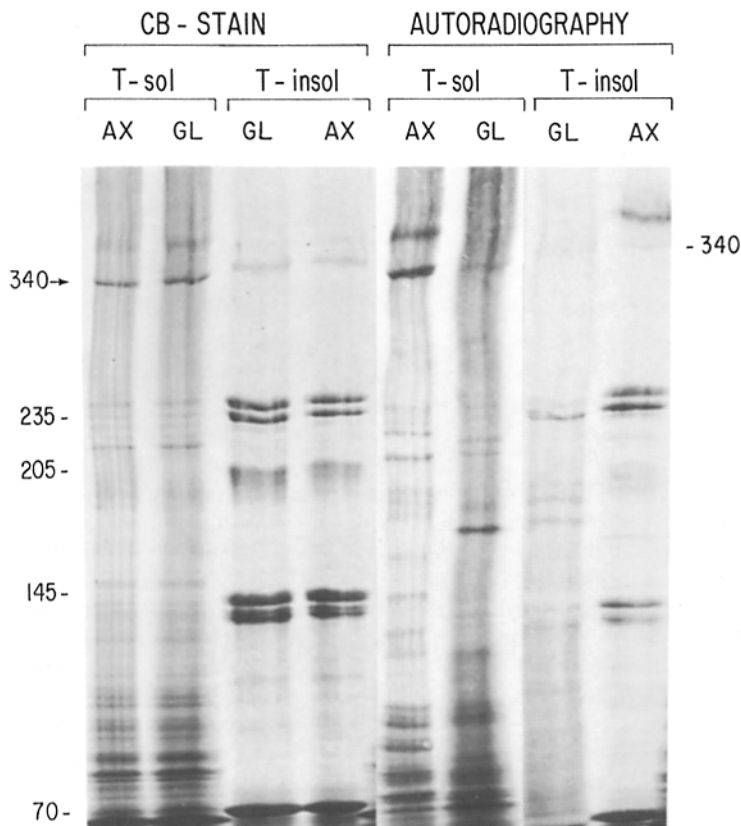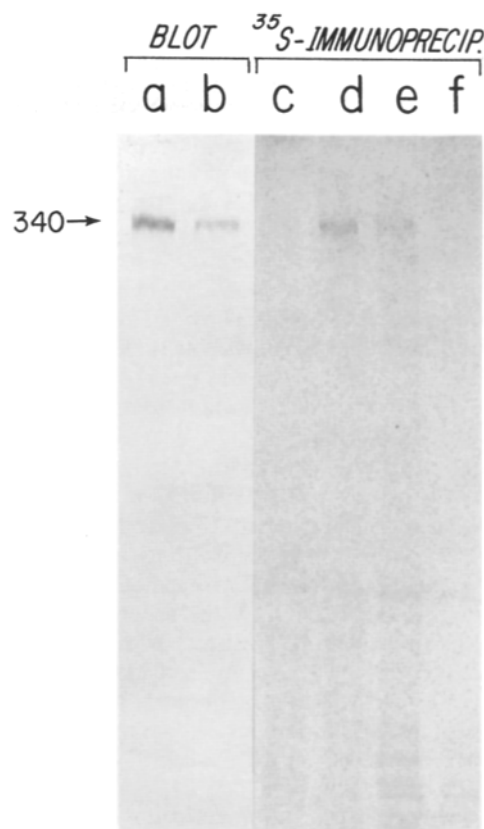

Figure 2. Comparison of 340-kD protein synthesized in optic glia and retinal ganglion cells. The SDS-PAGE (3–7% gradient) profiles of Triton-soluble (*T-sol*) and Triton-insoluble (*T-insol*) proteins from optic nerves stained with Coomassie blue (*CB-STAIN*) are compared with Triton-soluble and -insoluble proteins from optic nerves in which glial proteins (*GL*) or axonal proteins (*AX*) were selectively radiolabeled with [^{35}S]methionine as described in Materials and Methods. Labeled proteins were revealed by autoradiography. Triton-soluble fractions (115 μg protein) and Triton-insoluble fractions (77 μg protein) for each lane were derived from three optic nerves.
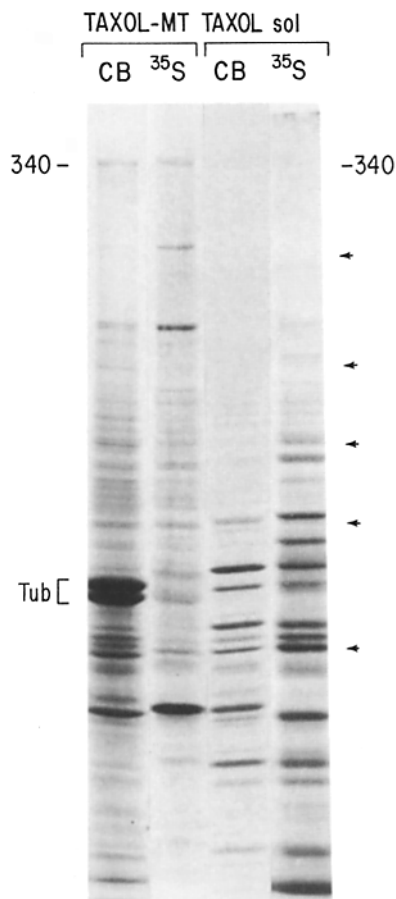
Figure 3. SDS-PAGE analysis of microtubule-enriched fractions from radiolabeled optic axons prepared in the presence of taxol (*TAXOL-MT*) compared with soluble proteins that did not co-assemble with microtubules (*TAXOL-sol*). Both fractions were derived from the same 30,000 g supernatant prepared from two radiolabeled optic pathways and were loaded onto gels in their entirety. Coomassie blue–stained patterns (*CB*) are compared with the corresponding autoradiograms of the ^{35}S-labeled axonal proteins (^{35}S). Radiolabeled optic axons were obtained from mice 3 d after intravitreal injection of [^{35}S]methionine. The positions of the 340-kD polypeptide and tubulin are indicated on these 5–15% polyacrylamide gels. Arrows indicate relative position of molecular mass standards in kilodaltons. (Top to bottom) Myosin, 210; β-galactosidase, 130; phosphorylase *b*, 94; BSA, 68; and ovalbumin, 43.

Figure 4. Identification of MAP 1A in optic axons by immunoprecipitation and immunoblotting. Optic axons labeled by intravitreal injection of [^{35}S]methionine 7 d before analysis were immunoprecipitated using a panel of anti–MAP 1 antibodies and then resolved by a 4–10% SDS-PAGE. (Lanes *c* and *f*) Anti–MAP 1B, clone 1B-5 (Bloom et al., 1984) and clone MAP 1/2 (Safaei and Fischer, 1989), respectively. (Lanes *d* and *e*) Anti–MAP 1A, clone 1A-1 (Bloom et al., 1984) and clone M1.4 (Brown and Binder, 1989), respectively. Optic axons and whole mouse brain homogenates (lanes *a* and *b*, respectively) were immunoblotted using anti–MAP 1A, clone 1A-1.

Triton-insoluble fractions. These fractions were subjected to SDS-PAGE, staining with Coomassie blue, and autoradiography. In comparison to the heavy labeling of a 340-kD protein in axons (Fig. 2), only a minor radioactive band was seen in the same region of gel lanes containing Triton-soluble, but not Triton-insoluble fractions of radiolabeled glial proteins (Fig. 2) indicating the possibility of a low level of expression of the 340-kD protein in glial cells.

The properties of the 340-kD protein were studied further by preparing a microtubule-enriched fraction by the taxol method from a 30,000 g supernatant fraction of [^{35}S]methionine-labeled retinal ganglion cell axonal proteins. The 340-kD polypeptide in both radiolabeled and unlabeled form, as well as several other lower molecular weight proteins, pref-

erentially associated with the fraction containing assembled tubulin (Fig. 3). Similar results were obtained when the microtubule-enriched fractions were prepared by co-assembly with purified brain microtubules (data not shown). These results suggested that the 340-kD protein may be a MAP 1–related polypeptide.

Since MAP 1 denotes a group of immunologically and structurally distinct polypeptides (Bloom et al., 1984), we established the relationship of the 340-kD protein to specific forms of MAP 1 by immunoblot and immunoprecipitation experiments using previously well-characterized antibodies to MAP 1A and MAP 1B. Proteins in optic axons were labeled in vivo with [^{35}S]methionine and the homogenates were immunoprecipitated with a panel of MAP 1 specific antibodies (Fig. 4). The 340-kD polypeptide was immunoprecipitated by two antibodies that recognize MAP1A: clone MAP 1A-1 (Bloom et al., 1984) and clone MAP M1.4 (Brown and Binder, 1989) as shown in Fig. 4 (lanes *d* and *e*, respectively). By contrast, no proteins were immunoprecipitated with two antibodies against MAP 1B: clone MAP 1B-5 (Bloom et al., 1984) and clone MAP 1/2 (Safaei and

Fischer, 1989) (Fig. 4, lanes c and f). Furthermore, immunoblot experiments with an antibody against MAP 1A confirmed the identity of the protein and showed that the optic nerve contained the high molecular weight form of MAP 1A which is equivalent to MAP 1A from whole mouse brain homogenates (Fig. 4, lane a and b). In addition, radiolabeled optic axons were immunoprecipitated with a panel of anti-MAP 1 antibodies as described in Fig. 4, resolved by a 4–10% SDS-PAGE, transferred to a nitrocellulose membrane and immunostained with clone MAP 1A-1 (Bloom et al., 1984). The immunoprecipitated MAP 1A protein detected by autoradiography of the nitrocellulose blot was identical to that of the radiolabeled 340-kD polypeptide and the MAP 1A-immunoreactive band from the soluble fraction of optic axons homogenates (data not shown).

Supporting the identification of the 340-kD polypeptide as MAP 1A and not MAP 1B were additional immunoblot studies of MAP expression in developing optic nerve that showed the levels of MAP 1B immunoreactivity to be relatively high at early phases of postnatal development (P8) and to decrease to almost undetectable levels in the adult mouse (data not shown). This pattern is consistent with previous reports on the changes in the relative levels of MAP 1B, or related proteins, in developing brain (Bloom et al., 1985; Calvert and Anderton, 1985; Riederer and Matus, 1985; Safaei and Fischer, 1989; Schoenfeld et al., 1989).

Axoplasmic Transport of MAP 1A in Relation to Tubulin

Since the ability of MAP 1A to bind to microtubules (Hirokawa, 1982; Shiomura and Hirokawa, 1987a,b) implies a possible functional interrelationship, we investigated whether or not the rate of axonal transport of MAP 1A was the same as that of tubulin subunits in optic axons by analyzing the change in distribution of these polypeptides along optic axons at three and seven days after injecting mice intravitreally with [35S]methionine. Radiolabeled optic axons were cut into nine consecutive 1.1-mm segments. The 100,000 g supernatant pool of proteins from each segment was assembled with taxol to yield a fraction enriched in microtubules and a fraction of soluble axonal proteins that do not assemble with tubulin. The two fractions for each of nine segments at two postinjection timepoints were analyzed by SDS-PAGE and autoradiography (Fig. 5). A comparison of the distribution of radiolabeled MAP 1A along optic axons at three and seven days after [35S]methionine injection indicated translocation of MAP 1A from proximal segments to more distal segments of optic axons (Fig. 5).

The distribution of tubulin subunits at these time points differed considerably from the patterns for MAP 1A. At 7 d after [35S]methionine injection, radiolabeled α- and β-subunits of tubulin were detected primarily in the proximal 2–3 mm of the optic pathway (Fig. 5). By contrast, MAP 1A radioactivity was highest 6–7 mm downstream from the eye. Autoradiograms of corresponding fractions from this experiment, which contained the radiolabeled axonal proteins that do not assemble with tubulin in the presence of Taxol, displayed no detectable radioactivity at the migration position of MAP 1A on SDS gels (data not shown).

Observations that labeled soluble tubulin was localized exclusively to the first three nerve segments in Fig. 5 were confirmed and extended in two-dimensional SDS-gel studies
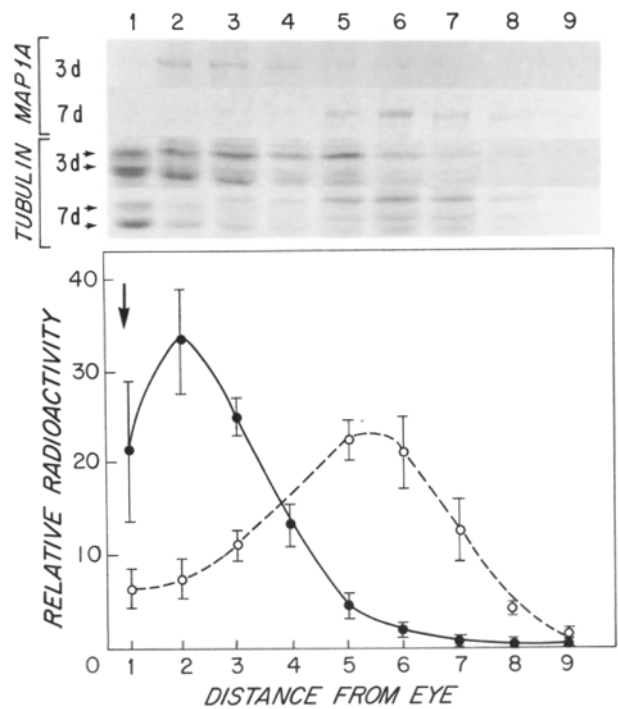

Figure 5. Distribution of taxol assembled MAP 1A and tubulin along consecutive 1.1-mm segments of the optic nerve and optic tract obtained 3 or 7 d after intravitreal injection of [35S]methionine. For each segment, microtubule-enriched insoluble fractions prepared in the presence of taxol were subjected to SDS-PAGE (5–15% acrylamide gradient), and labeled proteins were visualized by autoradiography. The MAP 1A region and corresponding tubulin region from the same gel are depicted. The graph depicts axoplasmic transport distribution profiles for MAP 1A at 3 (●) and 7 (○) d after intravitreal [35S]methionine injection are shown. Each point represents the mean ± SEM for determinations on three to four sets of unfractionated optic pathways, each representing the pooled tissue from five to nine mice. The position of the tubulin peak radioactivity at 3 and 7 d is indicated by the single arrow. The position of the peak did not change during this short interval.

of total (soluble and insoluble) tubulin distribution. Consecutive 1.1-mm segments of unfractionated optic pathways were obtained from mice 6 d after intravitreal injection of [3H]proline (Fig. 6). Densitometry of the α- and β-tubulin region of the fluorograms depicted in Fig. 6 indicated that 99% of the detectable tubulin radioactivity was present in segments one to three (69, 23, and 7%, respectively). Labeled 50–60-kD polypeptides that were unrelated to tubulin based on previous studies (Brown et al., 1982) moved at Group IV velocities and accounted for the presence of the 50–60-kD radioactive bands seen in distal nerve segments in Fig. 5.

To establish the rates of axoplasmic transport of MAP 1A and tubulins more precisely, we analyzed the changes in the distribution of these proteins in consecutive 1.1-mm segments of the optic axons at various intervals between 0.5 and 120 d after injection of [35S]methionine or [3H]proline. Since MAP 1A appears to be the only radiolabeled protein migrating at 340-kD on the gels, it can be identified and analyzed unambiguously. At each postinjection timepoint, the distributions of radioactivity associated with MAP 1A and
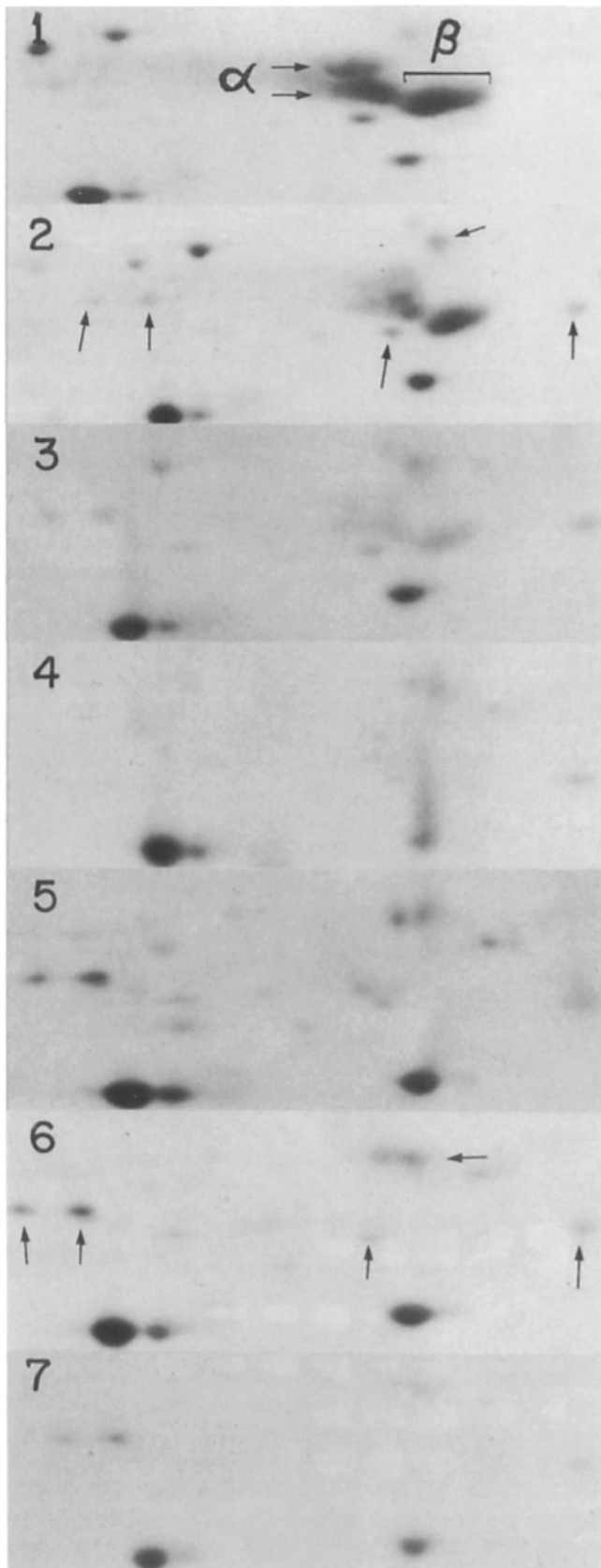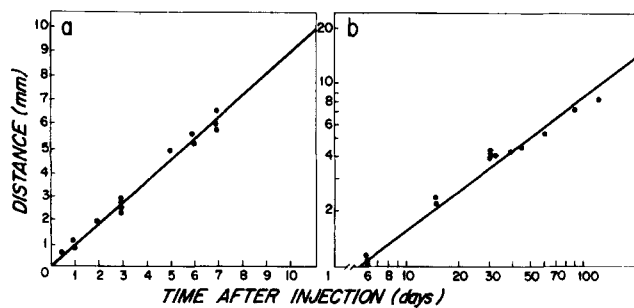
Figure 7. Regression analyses of movement of the transport waves of MAP 1A (*a*) or tubulins (*b*) as the function of time after intravitreal injection of [^{35}S]methionine or [^{3}H]proline. Consecutive 1.1-mm segments of mouse optic pathways were analyzed by SDS-PAGE between 0.5 and 120 d after isotope injection. At each timepoint, the distributions of radioactivity associated with MAP 1A and tubulins were plotted (e.g., Fig. 5) to identify the peaks of the moving transport wave for each protein. The distance of the peak from the eye was regressed against the time after isotope administration. Each point represents one complete analysis.

the tubulin subunits were quantitated and plotted to identify the peaks of the moving transport wave for each protein. The rate at which the wave moved was determined by regressing the distance of the wave peak from the eye against the time after isotope administration (Fig. 7). These regression curves indicated that MAP 1A advanced at a rate of 1.1-mm/d, a typical Group IV transport rate (Grafstein and Forman, 1980). By contrast, both tubulin subunits advanced at a rate of 0.1–0.2 mm/d, the characteristic rate of the Group V transport phase (Grafstein and Forman, 1980). This rate corroborates a previous estimate based upon a two-dimensional gel analysis of tubulin transport in mouse optic axons (Brown et al., 1982).
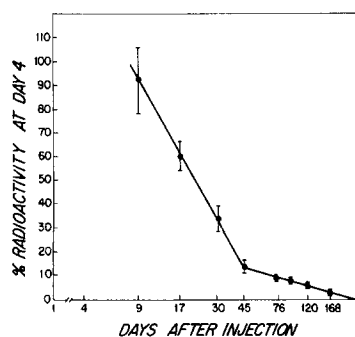
Kinetics of MAP 1A Disappearance from Axons

The apparent association of MAP 1A with microtubules in vitro (see review, Olmsted, 1986) and by immunoelectron microscopic analysis (Hirokawa, 1982; Shiomura and Hirokawa, 1987*a*,*b*) makes it difficult to reconcile the finding of different transport rates for tubulin and MAP 1A with a model in which each of these pools are continuously moving. Therefore, to examine the possibility that a portion of the transported MAP 1A contributes to a stationary cytoskeleton and thus can interact with stationary phase microtubules, we investigated the long-term fate of newly synthesized MAP 1A as it moved through a 9-mm length of the mouse optic pathway extending from the proximal optic nerve to the distal optic tract. The rate at which proteins enter and exit this axonal compartment reflects primarily their axoplasmic transport rate (Nixon and Logvinenko, 1986). In addition, if the initial specific radioactivity of MAP 1A synthesized in retinal ganglion cells is similar in groups of mice allowed to survive for different postinjection intervals, it is also possible to quanti-

Figure 6. Two-dimensional SDS-PAGE analysis of total (soluble plus insoluble) radiolabeled tubulin in seven consecutive 1.1-mm segments of unfractionated optic pathway from mice 6 d after intravitreal injection of [^{3}H]proline. Radiolabel associated with the α- and β-tubulins, detected by fluorography, is apparent in the first

three 1.1-mm segments, confirming the distribution of soluble tubulin on one-dimensional gels seen in Fig. 5. A group of minor labeled 50–60 kilodalton Group IV polypeptides unrelated to tubulin is designated by arrows in segments *2* and *6*.

tate the proportion of MAP 1A that is retained in axons longer than would be predicted from its axoplasmic transport rate, as demonstrated previously for neurofilament proteins (Nixon and Logvinenko, 1986).

In each of two experiments, 80–100 mice were injected intravitreally with [³H]proline and analyzed at 11 different intervals from 1 to 168 d after injection. Changes in the radioactivity associated with MAP 1A in the 9-mm length of optic axons were quantitated after SDS-PAGE in each of 8–16 mice per timepoint. MAP 1A began to disappear from optic axons at 8 d after injection. When plotted as a logarithmic function of time, the loss of radiolabeled MAP 1A from optic axons between 8 and 168 d was biphasic (Fig. 8). The time of initial MAP 1A disappearance, calculated by extrapolating the first slope in Fig. 8 to 100%, occurred at 8 d, indicating that the leading edge of the MAP 1A wave advanced at a rate of 1.1 mm/d, which is identical to the transport rate calculated in Fig. 7 using a different method. The first phase of disappearance between 8 and 45 d, referred to as phase I, occurred with a half-life of 12 d. Radiolabeled MAP 1A remaining in axons beyond 45 d (phase II) disappeared at a much slower rate ($t_{½} = 55$–60 d). The MAP 1A radioactivity retained in axons at 45 d after injection (phase II) was 13% of the peak MAP 1A radioactivity present at day 4 when radioactivity levels are highest. This represents a minimal estimate, since extrapolation of the Phase II values to correct for MAP 1A that has disappeared between 8 and 45 d gave a value of 27%. The residence time of MAP 1A in phase II, however, was 4.6 times longer than that of MAP 1A in phase I. Therefore, we estimated that under steady-state conditions at least 44% of the transported MAP 1A is associated with phase II (Table I).

In separate experiments, the distribution of the radiolabeled MAP 1A that is retained in axons after the main wave of transported MAP 1A has exited was determined by analyzing consecutive 1.1-mm segments of optic axons by SDS-PAGE and fluorography (Fig. 9). The MAP 1A band on autoradiograms of axonal proteins analyzed at intervals from 1 to 120 d after [³H]proline injection was distinctly observed and was among a minority of slowly transported pro-

Table I. Estimated Proportion of MAP 1A Associated with the Stationary Cytoskeleton along Optic Axons

Pool	Total pulse-labeled MAP 1A*	Half-life in axons‡	Relative half-life	Relative proportions in the steady-state§	Total MAP 1A in axons
	%	d			%
Phase I (moving)	87	13	1.00	87	56
Phase II (stationary)	13	60	4.62	60	44

* Calculated from the inflection point of the curve describing the biphasic disappearance of MAP 1A in Fig. 8.
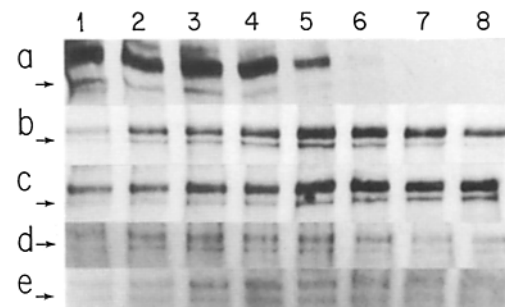‡ Calculated from the slopes of lines describing the two phases of MAP disappearance in Fig. 8.
§ Calculated by multiplying the size of the pulse-labeled MAP pool (column 2) by the relative half-life of the corresponding pool (column 3).

teins that were still significantly radiolabeled. Advance of the radiolabeled MAP 1A distally was evident until day 15 after isotope injection (Fig. 9, a–c). Although the absolute level of radioactivity associated with MAP 1A decreased from 45 to 120 days, the relative distribution of this label along optic axons remained the same (Fig. 9, d and e, and Fig. 10). This distribution paralleled the axonal distribution of the total content of MAP 1A, determined by densitometric analysis of Coomassie-stained gels containing consecutive 1.1-mm segments of the optic pathway (Fig. 10).

Discussion

Axonally Transported 340-kD MAP Is MAP 1A

The results of this study provide the first evidence for the synthesis and axoplasmic transport of a high molecular weight MAP in neurons in vivo. The identification of the transported polypeptide as MAP 1A is based upon co-migration on SDS gels with MAP 1A from brain microtubules at a characteristic electrophoretic mobility (340 kD); its ability to co-assemble with microtubules during cycles of assembly-disassembly; and recognition by specific antibodies

Figure 8. Relative proportions of newly synthesized MAP 1A entering and leaving a 9-mm axonal window. In each of two experiments, 80–100 mice were injected intravitreally with 25 μCi [³H]proline. At different intervals after injections, optic pathways were dissected from groups of these mice. After SDS-PAGE, the radioactivity associated with MAP 1A was quantitated and expressed as a percentage of the radioactivity present in the tissue sample at day 4 after injection. Each point is the mean ± SEM (error bars) for 8–18 determinations. Mean radioactivity in MAP 1A at day 4 was 70,486 dpm.

Figure 9. SDS-PAGE analysis of the distribution of radiolabeled MAP 1A along consecutive 1.1-mm segments of optic pathways from mice at different intervals after intravitreal injection of [³H]proline. Radiolabeled proteins were visualized after fluorography and exposure times were varied for optimal comparison. The distribution profiles are shown for postinjection intervals of 3, 7, 15, 45, and 120 d (a–e, respectively). The position of MAP 1A is indicated by arrows.
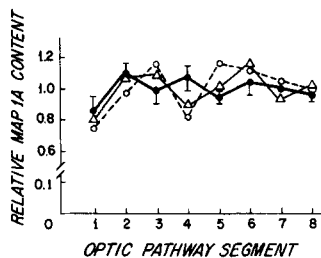
Figure 10. Distribution of MAP 1A along mouse optic axons. Optic pathways pooled from six to nine mice cut into eight consecutive 1.1-mm segments were subjected to one-dimensional SDS-PAGE. Proteins on the gels were stained with Coomassie blue, and the dye-binding capacity of the MAP 1A band, which reflects relative protein content, was determined by laser densitometry. Total protein was determined for each segment pool, and MAP 1A content in arbitrary units was expressed as a fraction of the total protein. Each point (●) is the mean ± SEM (error bars) for six determinations. For comparison, the distributions of radiolabeled MAP 1A at 30–45 (Δ; $n = 3$) or 120 (○; $n = 1$) d after [^{3}H]proline injection were determined by densitometry of fluorograms.

against MAP 1A in immunoblotting and immunoprecipitation assays. Experiments using a panel of well-characterized antibodies (Bloom et al., 1985) further identified the 340-kD MAP as MAP 1A. Immunocytochemical studies indicate that MAP 1A forms are present in neurons, including both the axonal and dendritic compartments; however, their presence in glial cells and nonneural tissues is controversial (Riederer et al., 1986; Bloom et al., 1984; Herrmann et al., 1985; Schoenfeld et al., 1989). In this regard, synthesis of MAP 1A in retinal ganglion cells greatly exceeded the level of incorporation of label into adjacent glial cells under conditions where proteins common to both cell types, such as fodrin, showed comparable degrees of radiolabeling (Nixon, 1986).

Consistent with previous reports on the expression of MAP 1A during development (Bloom et al., 1984; Riederer and Matus, 1985; Safaei and Fischer, 1989), levels of the 340-kD polypeptide in retinal ganglion cells are relatively high in the adult. By contrast, MAP 1B, or the equivalent forms of MAP 5 (Riederer et al., 1986), MAP 1 (Calvert and Anderton, 1985) and MAP 1.2 (Asai et al., 1985), which are present in relatively high levels early in brain development, decrease significantly in the adult (Bloom et al., 1985; Riederer et al., 1986; Calvert and Anderton, 1985). Similarly, MAP 1B was prominent on immunoblots of optic nerve proteins of mice at early postnatal ages and decreased to undetectable levels in optic axons of adult mice.

MAP 1A Is Transported Independently of Tubulin or Microtubules along Axons

MAPs have been shown to associate tightly with microtubules during assembly experiments in vitro and by immunocytochemical staining of cells and brain tissue (for review, Olmsted, 1986). Immunoelectron microscopy studies of neuronal cytoskeleton architecture specifically show that MAP 1A is localized to microtubules (Hirokawa, 1982; Hirokawa et al., 1985; Shiomura and Hirokawa, 1987a,b), suggesting a functional interrelationship in vivo. These studies demonstrated that MAP 1A extended outward from the microtubule wall as thin filaments apparently connecting microtubules to each other. It is significant, therefore, that MAP 1A is transported along optic axons at a different rate than tubulin. The rate of tubulin transport determined in this study (0.1–0.2

mm/d) is a typical Group V transport rate and agrees with studies in various neuronal systems which show that newly synthesized tubulin in CNS axons moves in the slowest phase of axoplasmic transport (Brown et al., 1982; Black and Lasek, 1980; Oblinger et al., 1987). In motor and sensory neurons of peripheral nerves (Mori et al., 1979; McQuarrie et al., 1986; Tashiro et al., 1984), a substantial proportion of newly synthesized tubulin moves at a Group IV rate. A faster moving component of radiolabeled tubulin, however, has not been detected in retinal ganglion cells (Brown et al., 1982; Black and Lasek, 1980; Oblinger et al., 1987).

Most, if not all, of the newly synthesized MAP 1A in retinal ganglion cells is transported at a rate of 1.0–1.2 mm/day, which is characteristic of the Group IV phase of axoplasmic transport in this neuronal system. This rate, determined by the conventional method of measuring changes in the advance of the moving wave of radiolabeled MAP 1A along axons, was confirmed by an independent method (Nixon and Logvinenko, 1986) based upon the arrival of MAP 1A at a distal site along optic axons (i.e., the end of the axonal window) and its rate of movement past this site. The constant rate of disappearance of radiolabeled MAP 1A from the axonal window between 8 and 45 d after injection of radiolabeled amino acid implies a single axonal transport rate for most of the newly synthesized MAP 1A pool. The axonal transport of MAP 1A differs from the behavior of the tau group of MAPs which advance along optic axons in the Group V wave of axoplasmic transport (Tytell et al., 1984). Our preliminary studies corroborate these findings by identifying several polypeptides in the 50–60-kD range that co-assemble with microtubules, selectively immunoprecipitate with anti-tau antibodies, and advance along axons at the characteristic Group V rate (Fischer and Nixon, unpublished data). Further studies, however, indicate that, with respect to axonal transport, tau MAPs are atypical when compared to the group of radiolabeled proteins in axons that co-assemble with microtubules. The majority of MAPs are synthesized and transported along optic axons in Group IV (Fischer, I., and R. A. Nixon, unpublished data).

MAP 1A Contributes to a Stationary Axonal Cytoskeleton: Functional Correlates

If the association between MAP 1A and microtubules shown biochemically and viewed ultrastructurally is a physiological interaction, the different rates of transport of MAP 1A and tubulin imply that some or all of the tubulin and MAP 1A must at least transiently move at the same rate or else become stationary during their residence within axons. No evidence for the former possibility exists; however, our studies indicate that a substantial proportion of the newly synthesized transported MAP 1A deposits into a stationary network along axons.

Although 85–90% of the pulse-radiolabeled MAP 1A continuously moved toward axon terminals in Group IV, the remainder was retained along optic axons for long periods after kinetic analyses predicted the complete elimination from axons of the MAP 1A in Group IV. Re-use of radiolabeled amino acids from axonal proteins undergoing proteolysis during transport could not account for the retained radiolabeled MAP 1A since glial cells synthesized relatively low levels of MAP 1A. Similarly, synthesis of MAP 1A from re-used [^{3}H]proline in ganglion cell perikarya and subse-

quent axonal transport of this labeled polypeptide is too minor to account for the levels of radiolabeled MAP 1A retained in axons at long intervals after intravitreal injection (Nixon and Logvinenko, 1986). The retained fraction is also unlikely to represent a co-migrating polypeptide unrelated to MAP 1A for several reasons. Due to its long half-life, it would represent more than half of the steady-state level of protein at the ~340 kD position on gels. A polypeptide of this abundance would be easily resolved from MAP 1A in our studies (Figs. 1, 3, and 4) unless the protein were to be previously undescribed MAP that migrates identically to MAP 1A on high resolution SDS gels.

Once the moving wave of radiolabeled MAP 1A reached distal ends of optic axons by 12–15 d after injection, the radiolabeled MAP 1A persisting along axons remained uniformly distributed at proximal and distal axonal levels, mimicking the distribution of unlabeled MAP 1A. The constancy of this distribution pattern over long time intervals after isotope injection indicated that this fraction of radiolabeled MAP 1A was stationary in axons. The fraction of MAP 1A retained in axons was at least 13% of the total pulse-radiolabeled MAP 1A, and the residence time of this pool of labeled molecules was 4.6 times longer than that of the MAP 1A in Group IV. Thus, under steady-state conditions, nearly half of the MAP 1A in optic axons is associated with stationary axonal structures. These calculations emphasize the importance of considering not only the relative radioactivity of each pool of a particular pulse-labeled protein but also the rates of turnover of each when determining the relative sizes of the pools in the steady state.

The existence of a stationary pool of MAP 1A has as a precedent the earlier findings that axonally transported neurofilaments are also incorporated into a stationary neurofilament network along optic axons (Nixon and Logvinenko, 1986). Various other cytoskeleton-associated proteins also contribute to the stationary axonal cytoskeleton (Nixon, R. A., unpublished observations). The existence of a stationary population of axonal microtubules, in particular, is supported by increasing experimental evidence from other laboratories. Tubulin subunits are added at the distal tip of the neurite of cultured neurons and primary cultures of dorsal root ganglion cells (Bamburg et al., 1986; Letourneau and Ressler, 1984) consistent with the observation (Heidemann et al., 1981) that the sites of subunit addition on microtubules (+ end) are oriented exclusively distal to the cell body (Schulze and Kirschner, 1987; Sammak et al., 1987). In neurites of PC12 cells, biotinylated tubulin dimers microinjected into the cell body incorporated mainly at the distal ends of neurites. Immunoelectron microscopy confirmed that free tubulin subunits reached distal sites along neurites and became locally incorporated into the neurite cytoskeleton (Okabe and Hirokawa, 1988). Recently, photobleached zones of fluorescently labeled microtubules in PC12 cells were shown not to change position along the neurite, indicating that these microtubules were stationary (Lim et al., 1989). Finally, in several neuron systems, a proportion of labeled newly synthesized tubulin persists in proximal regions of axons after the moving wave has passed (Watson et al., 1988; Nixon, R. A., unpublished data).

The dynamics of MAP 1A transport and deposition relative to behavior of tubulin supports the concept that a major part of the cytoskeleton assembles locally within axons as in-

dividual transported cytoskeletal elements, either in the form of subunits or polymers, leave the transport vector (Nixon, 1987, 1990; Hollenbeck, 1989). The relatively uniform distribution of tubulins (Brown et al., 1982) and MAP 1A differs from the increasing proximal-to-distal gradient of neurofilaments (Nixon and Logvinenko, 1986) and certain other cytoskeletal proteins (Nixon, R. A., unpublished data). These results accord with the differing stoichiometry of microtubules and neurofilaments at different levels along axons (Nixon, 1987) and extend previous evidence for regional specialization of the axonal cytoskeleton along its length (Brown et al., 1982; Nixon et al., 1982). The movement of neurofilament and microtubule proteins in the same transport phase has traditionally emphasized the notion of the neurofilament-microtubule lattice as a distinct functional unit. Microtubule and neurofilament proteins, however, move independently under a variety of conditions (for review, Nixon, 1987). Although extensively interconnected in axons, neurofilaments and microtubules also appear to be physically associated with various axonal polypeptides that move in other transport classes (Nixon, 1989). Therefore, it is possible that similarity in transport rate overemphasizes the functional interrelationship between neurofilaments and microtubules relative to that between these networks and other cytoskeletal structures.

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