Widespread Distribution of the Major Polypeptide Component of MAP 1 (Microtubule-associated Protein 1) in the Nervous System

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ABSTRACT   We prepared a monoclonal antibody to microtubule-associated protein 1 (MAP 1), one of the two major high molecular weight MAP found in microtubules isolated from brain tissue. We found that MAP 1 can be resolved by SDS PAGE into three electrophoretic bands, which we have designated MAP 1A, MAP 1B, and MAP 1C in order of increasing electrophoretic mobility. Our antibody recognized exclusively MAP 1A, the most abundant and largest MAP 1 polypeptide. To determine the distribution of MAP 1A in nervous system tissues and cells, we examined tissue sections from rat brain and spinal cord, as well as primary cultures of newborn rat brain by immunofluorescence microscopy. Anti-MAP 1A stained white matter and gray matter regions, while a polyclonal anti-MAP 2 antibody previously prepared in this laboratory stained only gray matter. This confirmed our earlier biochemical results, which indicated that MAP 1 is more uniformly distributed in brain tissue than MAP 2 (Vallee, R. B., 1982, J. Cell Biol., 92:435–442). To determine the identity of cells and cellular processes immunoreactive with anti-MAP 1A, we examined a variety of brain and spinal cord regions. Fibrous staining of white matter by anti-MAP 1A was generally observed. This was due in part to immunoreactivity of axons, as judged by examination of axonal fiber tracts in the cerebral cortex and of large myelinated axons in the spinal cord and in spinal nerve roots. Cells with the morphology of oligodendrocytes were brightly labeled in white matter. Intense staining of Purkinje cell dendrites in the cerebellar cortex and of the apical dendrites of pyramidal cells in the cerebral cortex was observed. By double-labeling with antibodies to MAP 1A and MAP 2, the presence of both MAP in identical dendrites and neuronal perikarya was found. In primary brain cell cultures anti-MAP 2 stained predominantly cells of neuronal morphology. In contrast, anti-MAP 1A stained nearly all cells. Included among these were neurons, oligodendrocytes and astrocytes as determined by double-labeling with anti-MAP 1A in combination with antibody to MAP 2, myelin basic protein or glial fibrillary acidic protein, respectively. These results indicate that in contrast to MAP 2, which is specifically enriched in dendrites and perikarya of neurons, MAP 1A is widely distributed in the nervous sytem.

Microtubules purified from a variety of sources have been shown to consist of both tubulin, the principal structural protein, and a diverse group of microtubule-associated proteins, or MAP (5, 6, 27, 34, 45, 46). The MAP have been implicated in the regulation of microtubule assembly (26, 46), and in mediating the interaction of microtubules with other structural elements of the cytoplasm (1, 3, 12, 13, 15, 22, 32, 33, 35).

In brain, the most prominent MAP are high molecular weight proteins. These proteins have been divided into two classes, MAP 1 and MAP 2, on the basis of their electrophoretic mobility (34). MAP 2 (M_r 270,000) remains soluble when microtubules are exposed to elevated temperature, a property that allows this protein to be readily separated from MAP 1 (14, 17). MAP 2 purified in this way is seen as a fine filamentous arm on the surface of microtubules and promotes

microtubule assembly in vitro. MAP 2 purified under less severe conditions contains low molecular weight subunits of M_r 70,000, 54,000, and 39,000 (42). The latter two of these proteins represent the regulatory and catalytic subunits of a type II cAMP dependent protein kinase, which is associated with the MAP 2 arm (36, 42) and which extensively phosphorylates MAP 2 itself (34, 37, 39). MAP 2 is unusually sensitive to digestion by chymotrypsin and trypsin, which results in the rapid release of the arm portion of the protein from the microtubule (39, 41). The protein binds to actin in vitro (13, 32) and to intermediate filaments in cultured cells (3).

Like MAP 2, MAP 1 (M_r ~350,000) appears as a filamentous arm of similar dimension on the microtubule surface (43), and promotes microtubule assembly (20, 43). MAP 1 is as sensitive to proteases as MAP 2 (41) and much of the mass of MAP 1, as well as MAP 2, is rapidly released from the microtubules during protease treatment. MAP 1 is as stable as MAP 2 to some denaturants (43), but is insoluble at elevated temperatures (14, 17). Like MAP 2, MAP 1 contains low molecular weight subunits, but these are distinct in molecular weight (M_r 28,000 and 30,000) and stoichiometry from those associated with MAP 2 (43). MAP 1 is only slightly phosphorylated in vitro compared with MAP 2 (34, 39). In addition to these distinctions, antibodies, to MAP 2, the smaller of the two proteins, have not been observed to react with MAP 1 (3, 16, 29).

Thus, it appears that while MAP 1 and MAP 2 are two members of a class of structural proteins associated with microtubules, they have several distinctive features. Presumably, these features indicate that the proteins are functionally distinct as well. In support of this contention we have recently obtained biochemical evidence that MAP 1 and MAP 2 are differentially distributed in microtubules isolated from calf cerebral cortex and corpus callosum (40). These two brain regions contain distinct cellular and subcellular elements. Neuronal cell bodies, dendrites and axons, as well as glial cells, are present in cerebral cortex gray matter, while only axons and glial cells are present in white matter. MAP 2 is most abundant in microtubules isolated from calf cerebral cortex, but is greatly diminished in microtubules isolated from calf corpus callosum. MAP 1, in contrast to MAP 2, is present in identical amounts in microtubules from the two brain regions. Consistent with the nonuniform distribution of MAP 2 is the observation that antibody prepared against MAP 2 intensely stains neuronal cell bodies and dendrites in rat brain gray matter, but fails to stain glial cells and axonal processes appreciably in white matter tracts (8, 24; see Figs. 3 and 8 in this paper). These observations indicate that MAP 2 has a relatively restricted cellular and subcellular distribution, consistent with observations on cultured cells (3, 16, 29), radioimmune assays of tissue extracts (38), and an immunocytochemical study using an antibody to unfractionated high molecular weight brain MAP (23). In the latter study immunoreactivity was not detected in white matter tracts, suggesting that neither MAP 1 nor 2 is present in axonal processes or nonneuronal cells of central nervous system tissue. This contrasts our own biochemical work described above (40), which indicates that MAP 1 is abundant in white matter.

In the present study, we sought to define the cellular and subcellular distribution of MAP 1 and to compare the distribution of this protein with MAP 2. To this end we have prepared a monoclonal antibody to MAP 1. We found that MAP 1 is a complex of electrophoretic bands that can be divided into three biochemically distinguishable species. Our antibody recognizes the most abundant of these species, to which we now refer as MAP 1A. Consistent with our earlier biochemical results (40), we find that the immunoreactive MAP 1 species is widespread in brain and spinal cord compared to MAP 2. In the accompanying paper (4) we survey a broad variety of cells derived from diverse tissues and show that MAP 1A is commonly found outside of the nervous system as well.

MATERIALS AND METHODS

Monoclonal Antibody Production: A BALB/c mouse was injected subcutaneously with MAP 1 on two occasions spaced 6 wk apart. The immunogen was isolated from microtubules prepared from calf brain cerebral cortex by three temperature-dependent assembly cycles (42). The microtubule proteins were separated by SDS PAGE on a 9% polyacrylamide slab gel, which was stained with Coomassie Brilliant Blue R250. A strip of the gel containing the three MAP 1 bands (see Results) was excised, homogenized in 20 mM sodium phosphate, pH 7.4/150 mM NaCl, and emulsified by sonication with an equal volume of Freund's complete adjuvant. 10 d after the second injection, the mouse was given a final intraperitoneal boost of native MAP 1 prepared from calf brain white matter (43).

4 d after the final injection, immune splenic lymphocytes were collected and fused (18) with P3-NS1/1-Ag4-1 (NS1) mouse myeloma cells in a solution containing RPMI 1640 (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY), 37% polyethylene glycol 1000 (Koch-Lite Laboratories, Ltd.; Colnbrook, Buckinghamshire, England) and 5% DMSO (Sigma Chemical Co., St. Louis, MO). Cells were plated in 24-well tissue culture dishes (Falcon; Becton Dickinson Labware, Oxnard, CA), where they were maintained in HAT selective medium for 24 h to 2 wk postfusion. Cells were cloned by limiting dilution in 96-well dishes (Falcon) in a medium containing RPMI 1640, 20% fetal calf serum (Hy-Clone; Sterile Systems, Logan, UT) and pen/strep (Gibco Laboratories). Stable clones were maintained in medium containing 10% fetal calf serum.

Hybridomas, both uncloned and cloned, were screened for antibody production by immunofluorescence microscopy of primary brain cell cultures (see below) using conditioned hybridoma medium. Immunoreactive proteins were subsequently identified by immunoblot analysis (see below), also using conditioned medium. Antibody isotypes were determined by double immunodiffusion (28) with rabbit antibodies specific for mouse antibody isotypes (Bionetics Laboratory Products, Kensington, MD).

Immunocytochemistry and Immunochemistry: Brain and spinal cord tissues were obtained from young adult (3–4-mo old) female rats. The animals were anesthetized with Nembutal and perfused transcardially with 2% paraformaldehyde, 1.5% picric acid in 0.15 M phosphate buffer at pH 7.3 before dissection of the tissue. Fixation was continued overnight in the same solution at room temperature. Vibratome sections (20 μm) were collected in PBS and processed in suspension for single or double immunofluorescence microscopy as we have described for cultured brain cells (3), with the following exceptions: the 10% normal serum (sheep or goat) step was performed overnight at 4°C in the presence of 0.25% Triton X-100; all subsequent solutions contained 0.025% Triton X-100; antibody incubations were at 4°C for 24–48 hr. Stained sections were mounted on gelatin-coated glass slides, coated with a thin film of elvanol mounting medium (10) and overlaid with glass coverslips. Primary cultures of newborn rat brain were prepared and processed for single or double immunofluorescence microscopy after methanol fixation as we have described (3).

Antibodies used for these studies were as follows: *First antibodies:* anti-MAP 2, 1A, monoclonal mouse IgG1, described above; anti-MAP 2, polyclonal rabbit antibody (3, 8, 24, 36); anti-glial fibrillary acidic protein, rabbit polyclonal antibody supplied by Dr. R. Liem (New York University School of Medicine); antimyelin basic protein, polyclonal rabbit antibody, supplied by Sheryl L. Preston and Dr. Arthur McMorris (Wistar Institute). *Second antibodies:* Fluorescein conjugated IgG fraction of goat anti-rabbit IgG was from Cappel Laboratories (Cochranville, PA). Rhodamine conjugated IgG fraction of sheep anti-mouse IgG was prepared in our laboratory by the procedure of Forni (9) from sheep antiserum to mouse IgG (Cappel Laboratories).

Nitrocellulose replicas of SDS gels were prepared and stained with either conditioned hybridoma medium or ascites fluid antibody using conditions designed to obtain efficient transfer of high molecular weight polypeptides (3). The second antibody was peroxidase-conjugated IgG fraction of sheep anti-mouse IgG (Cappel Laboratories), which was reacted with 4-chloro-1-naphthol (Sigma Chemical Co.) to reveal the immunoreactive proteins.

RESULTS

Electrophoretic and Immunochemical Analysis of MAP 1

An immunoblot of unfractionated rat brain proteins stained with the monoclonal IgG1 antibody used throughout this paper is shown in Fig. 1A. Note the specific staining of a protein at the position of MAP 1. MAP 1 was originally defined as a single electrophoretic component on high percent polyacrylamide gels (34), but multiple bands have been seen in the MAP 1 region on low percent gels (11). Fig. 1B shows high molecular weight brain MAP obtained from calf white matter (40). MAP 1 is more prominent than MAP 2 in these preparations, and we find that the electrophoretic composition of the MAP 1 region can be more readily analyzed than in microtubules purified from whole brain or cerebral cortex. Five polypeptides were readily distinguished in the MAP 1/MAP 2 region of the gel (Fig. 1B). The two most rapidly migrating polypeptides were soluble at elevated temperatures, and reacted with both polyclonal and monoclonal anti-MAP 2 antibodies prepared in our laboratory (44). These two polypeptides are therefore considered to be components of MAP 2 (14, 17), and we refer to them as MAP 2A and MAP 2B. We refer to the three remaining peptides of higher molecular weight as MAP 1A, MAP 1B, and MAP 1C in order of increasing electrophoretic mobility. MAP 1A was the most prominent of the three polypeptides in both gray matter (not shown) and white matter microtubules. The band was broad and was occasionally observed to split into two distinct bands,

which suggests that even this species may be heterogeneous. MAP 1B was the second most prominent band and it split on occasion. MAP 1B was more prominent in microtubules purified from white matter than from gray matter (not shown). The MAP 1C band was the least prominent of the three bands. This protein could be readily distinguished from MAP 1A, MAP 1B, and the MAP 2 bands by exposure of the microtubules to low levels of chymotrypsin. Under these conditions (Fig. 1C), all bands except MAP 1C were readily destroyed (39, 41).

Immunoblot analysis was performed on brain MAP to determine the specificity of the monoclonal anti-MAP 1 antibody prepared for this study. It may be seen (Fig. 1B and C) that the antibody recognized only MAP 1A, the major MAP 1 polypeptide. To indicate the specificity of the antibody, we refer to it in this and the following paper (4) as anti-MAP 1A. To determine whether the antibody recognized ankyrin, a major erythrocyte cytoskeletal protein reported to be immunologically related to MAP 1 (2), we used anti-MAP 1A to stain immunoblots of sheep erythrocyte ghosts and rat erythrocytes (not shown). The antibody did not react with the ankyrin present in either sample.

Localization of MAP 1A in Axons and Oligodendrocytes

In a previous biochemical study we found that MAP 1 and MAP 2 were present at different relative concentrations in microtubules purified from calf brain gray and white matter (40). MAP 2 was considerably more abundant in gray matter than in white. When sections of brain tissue were examined by immunofluorescence microscopy, MAP 2 was observed to be highly concentrated in the dendritic processes of neurons, and was undetectable in axonal tracts and glial cells (8, 24), consistent with the biochemical result. On the other hand, the ratio of MAP 1 to tubulin was identical in microtubules isolated from gray and white matter (40), suggesting a more nearly uniform distribution for this protein than for MAP 2. To evaluate the distribution of MAP 1 in tissues of the nervous system, we stained sections of tissue from rat brain and spinal cord with our anti-MAP 1A monoclonal antibody.

Fig. 2a shows a low magnification view of rat corpus callosum white matter and surrounding gray matter of the cerebral cortex stained with anti-MAP 1A. Immunoreactivity was almost uniformly distributed across the field. This result is consistent with our biochemical evidence that MAP 1 is found at equal concentrations in microtubules isolated from cerebral gray and white matter (40), and suggests that significant levels of MAP 1A may be found in axons or glial cells, as well as in dendrites. No staining was observed when unconditioned medium was used (Fig. 2b) in place of conditioned hybridoma medium containing monoclonal anti-MAP 1A. We found that major cerebral white matter tracts other than corpus callosum generally stained brightly with anti-MAP 1A, but extremely weakly with anti-MAP 2. An example of these staining patterns in the anterior commissure is illustrated in Fig. 3. Here, anti-MAP-1A reactivity (Fig. 3a) was substantial, and was similar to that observed in the adjacent gray matter (bed nucleus of stria terminalis). Anti-MAP 2 (Fig. 3b), in sharp contrast, hardly stained the anterior commissure, although apparent dendrites in the adjacent gray matter were brightly stained.

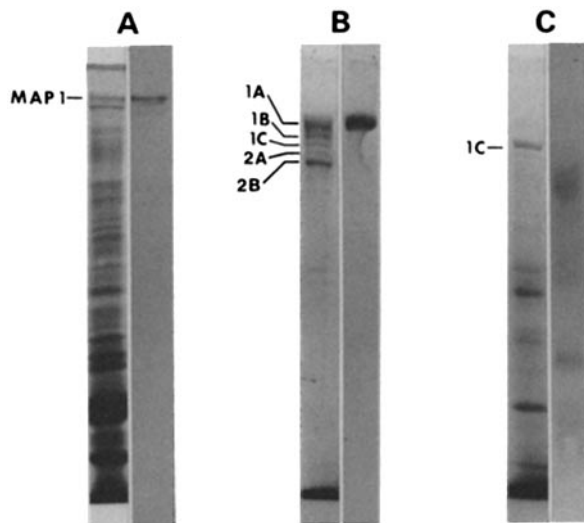The immunoreactivity of white matter with anti-MAP 1A

Figure 1 Electrophoretic and immunochemical properties of MAP 1. (A) A newborn rat brain was dissolved in sample buffer and an aliquot was subjected to SDS PAGE on a 7% polyacrylamide gel. Left, Coomassie Brilliant Blue R250; right, nitrocellulose replica stained with anti-MAP 1A (B) 4% gel of microtubules purified by the taxol procedure (40) from calf corpus callosum. Left, Coomassie Blue; right nitrocellulose replica stained with anti-MAP 1A. The positions of MAP 1A, 1B, 1C, 2A, and 2B are indicated. Note the specificity of the antibody for MAP 1A. (C) 4% gel of microtubules from calf corpus callosum (see B) treated for 10 min with 0.33 μg/ml chymotrypsin. (Lanes in B and C were adjacent in original gel and nitrocellulose replica.) Among the high molecular weight MAP, MAP 1C alone was resistant to proteolysis, indicating that this is a protein species distinct from MAP 1A and MAP 1B. Left, Coomassie Blue; right, nitrocellulose replica stained with anti-MAP 1A.
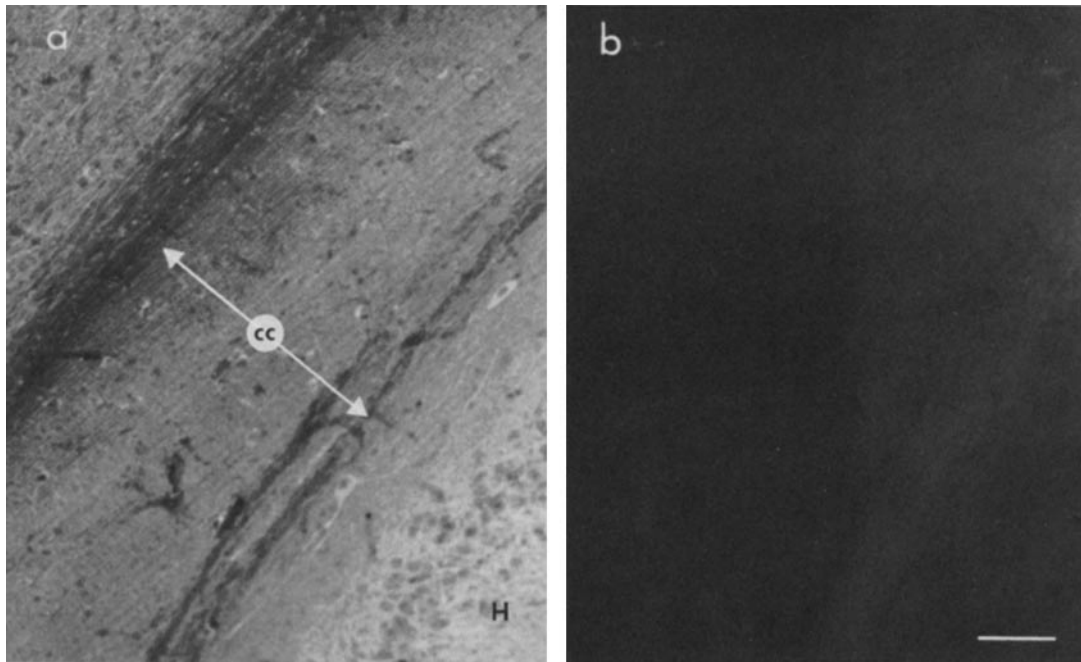
FIGURE 2   Localization of MAP 1A in rat cerebral white matter. (a) Sagittal section of corpus callosum white matter (cc) and adjacent gray matter of the cortex stained with anti-MAP 1A (conditioned hybridoma culture medium). A portion of the hippocampus (H) is visible in the lower right corner. (b) Control section processed for immunofluorescence microscopy and photographed identically to the section in a, except that unconditioned medium was used in place of conditioned hybridoma medium containing anti-MAP 1A. Bar, 50 μm. × 200.
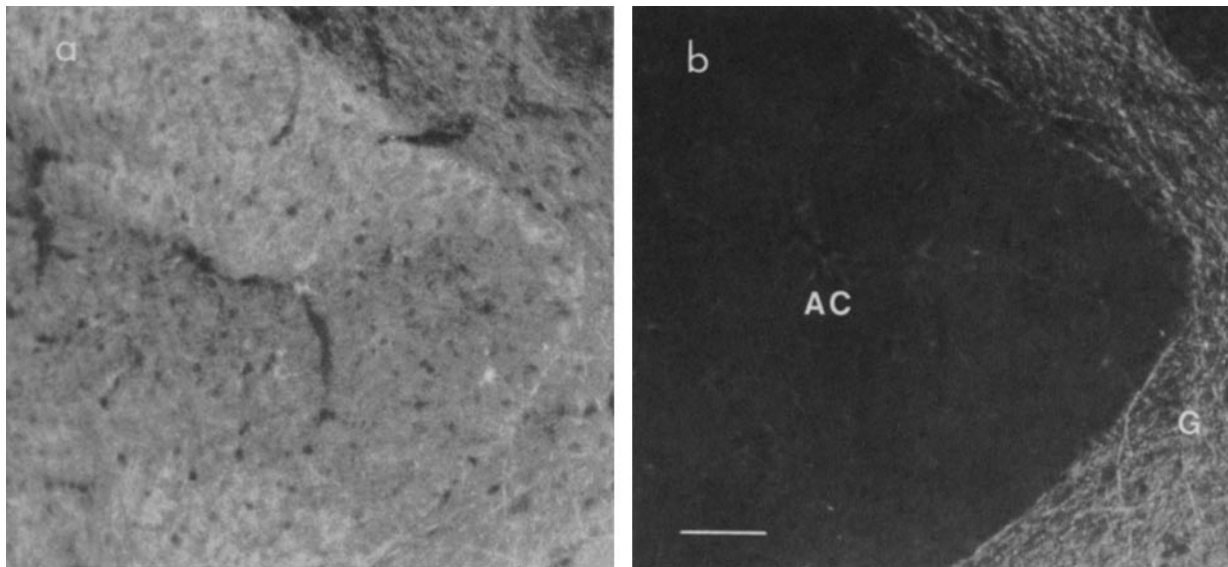
FIGURE 3   Comparison of MAP 1A and MAP 2 distributions in rat brain. A sagittal section of rat cerebrum was double-labeled with monoclonal anti-MAP 1A (a) and rabbit anti-MAP 2 (b) followed by rhodamine sheep anti-mouse IgG plus fluorescein goat anti-rabbit IgG. Visible in the section are a caudal portion of the anterior commissure (AC in b) plus adjacent gray matter (G in b; this region corresponds to the bed nucleus of the stria terminalis). Bar, 50 μm. × 240.

indicated that MAP 1A might be localized there in axons, glial cells, or both. Fine processes were generally labeled by the antibody in white matter regions, which could have reflected the presence of MAP 1A in either axonal processes or glial cell processes. The immunoreactivity of axons in particular was evident in certain regions. Fig. 4 shows a sagittal section of rat cerebrum stained with anti-MAP 1A. Numerous longitudinally cut bundles of axons in the immediate vicinity of the anterior commissure were observed to be intensely stained by the antibody. These fiber tracts ran from a ventral-caudal to a dorsal-rostral position and probably correspond to the striohypothalamic tract (19). In this region, the axonal staining was as prominent as the staining of dendrites and neuronal cell bodies in the surrounding tissue. Fig. 5 shows large myelinated axons in the dorsal root of a spinal nerve. Staining was observed in the axoplasm of these processes, and was absent in the myelin sheaths. The outer rim of some myelin sheaths was lightly stained and this may have repre-
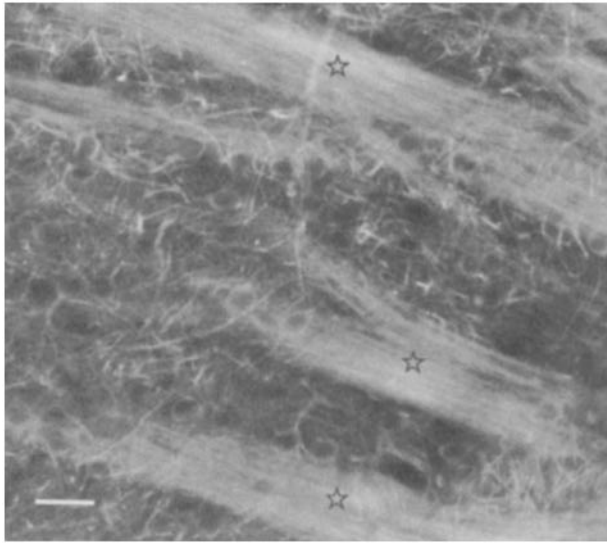
FIGURE 4 Localization of MAP 1A in axons. A sagittal section of the cerebrum was stained with anti-MAP 1A. The region shown is immediately rostral to the anterior commissure, which lies to the right of the field. The broad fiber bundles indicated by the stars are longitudinally cut axons, corresponding to the striohypothalamic tract, which is coursing through the nucleus accumbens. Bar, 20 µm. × 400.

sented thin regions of Schwann cell cytoplasm surrounding the sheath. Similar staining by anti-MAP 1A was observed in ventral roots of spinal nerves (not shown). Thus, in the peripheral nervous system MAP 1A was judged to be present in axons of identifiable sensory (dorsal root) and motor (ventral root) neurons.

In the spinal cord staining of myelinated processes was also observed (Figs. 5 and 6). In addition to axonal staining, anti-MAP 1A stained a class of cells that were scattered throughout white matter. At high magnification (Fig. 6, c and d) it could be seen that both broad and fine processes belonging to these cells were stained. These processes appeared to contact the outer layer of unstained myelin sheaths, and did not seem to contact axons directly. These histological properties are consistent with the identification of these cells as oligodendrocytes. Thus, it seemed that MAP 1A is present in both axons and oligodendrocytes, and may be especially concentrated in the latter.

Similarities in Anti-MAP 1A and Anti-MAP 2 Immunoreactivity

While MAP 1A was always detectable in white matter, the intensity of staining varied in different regions, as did the intensity relative to gray matter. In the cerebellum, for example (Fig. 7A), white matter staining was relatively weak compared to that observed in Purkinje cells. Thus, the overall staining pattern with anti-MAP 1A gave an impression similar to that observed with anti-MAP 2 (8), though distinct labeling of fibers in white matter was definitely detectable with antibodies to MAP 1A, but not to MAP 2. The somata and dendrites of the Purkinje cells were among the most prominently stained structures in the central nervous system (Fig. 7, A and B). Staining of Purkinje cell axons was also seen (Fig. 7B). In favorable sections weaker staining could be observed in granule cell and Golgi neurons in the granule

layer, and in basket cells in the molecular layer. Fine immunoreactive fibers (Fig. 7A) in the molecular layer may represent both dendrites and axons emanating from basket cell, Golgi, and granule cell neurons. Some of these processes were observed to cross the Purkinje cell body layer from the granule layer to the molecular layer. The fine caliber of these processes indicates they may be climbing fiber or parallel fiber axons.

Since both MAP 1A (Fig. 7) and MAP 2 (8, 24) were prominent in dendrites and neuronal cell bodies, this raised the question of whether the two proteins coexist in the same neuron. To resolve this point we double-labeled sections of brain and spinal cord with anti-MAP 1A and anti-MAP 2. Prominent, coincident labeling of large processes and neuronal perikarya in gray matter by both antibodies was generally observed. Fig. 8 shows examples of this from the spinal
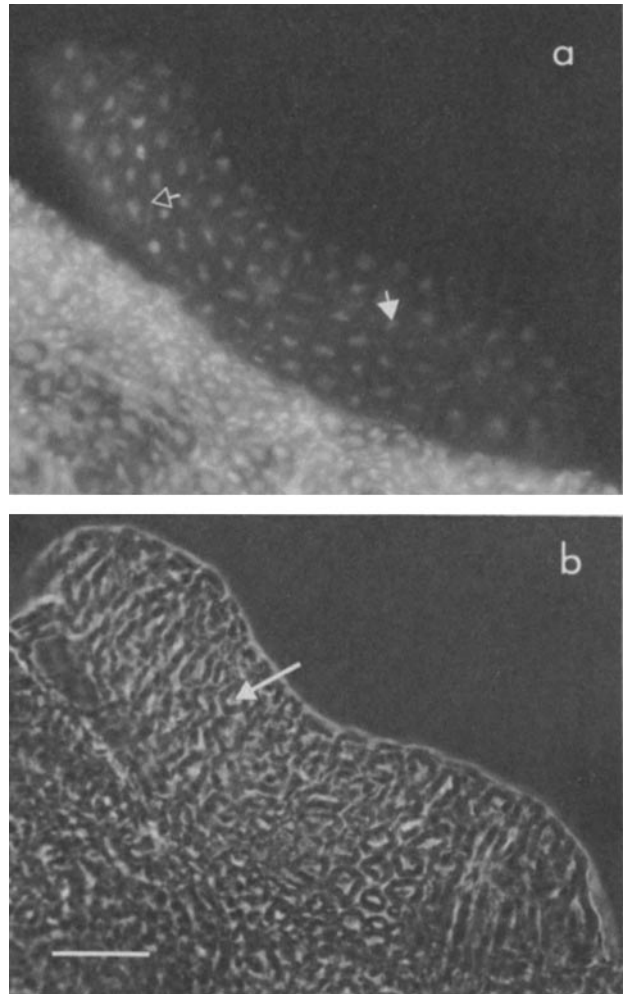

FIGURE 5 Axonal localization of MAP 1A in spinal nerve. A cross-section of the dorsal root of a spinal nerve in the cervical region is shown. (a) Anti-MAP 1A stained axoplasm (solid arrow), but not the surrounding myelin sheaths. Thin halos of immunoreactivity (open arrow) were occasionally observed surrounding the sheaths, and these may represent Schwann cell cytoplasm. The brightly stained area below the spinal nerve is spinal cord white matter, where both axons and oligodendrocytes are immunoreactive with anti-MAP 1A (see Fig. 6). (b) Phase-contrast image of the same field. Axons (solid, long-stemmed arrow) are visible as phase-lucent cores surrounded by phase-dense myelin sheaths. Phase-contrast image was printed darkly to optimize contrast between myelin sheaths and axonal cytoplasm. Bar, 20 µm. × 650.
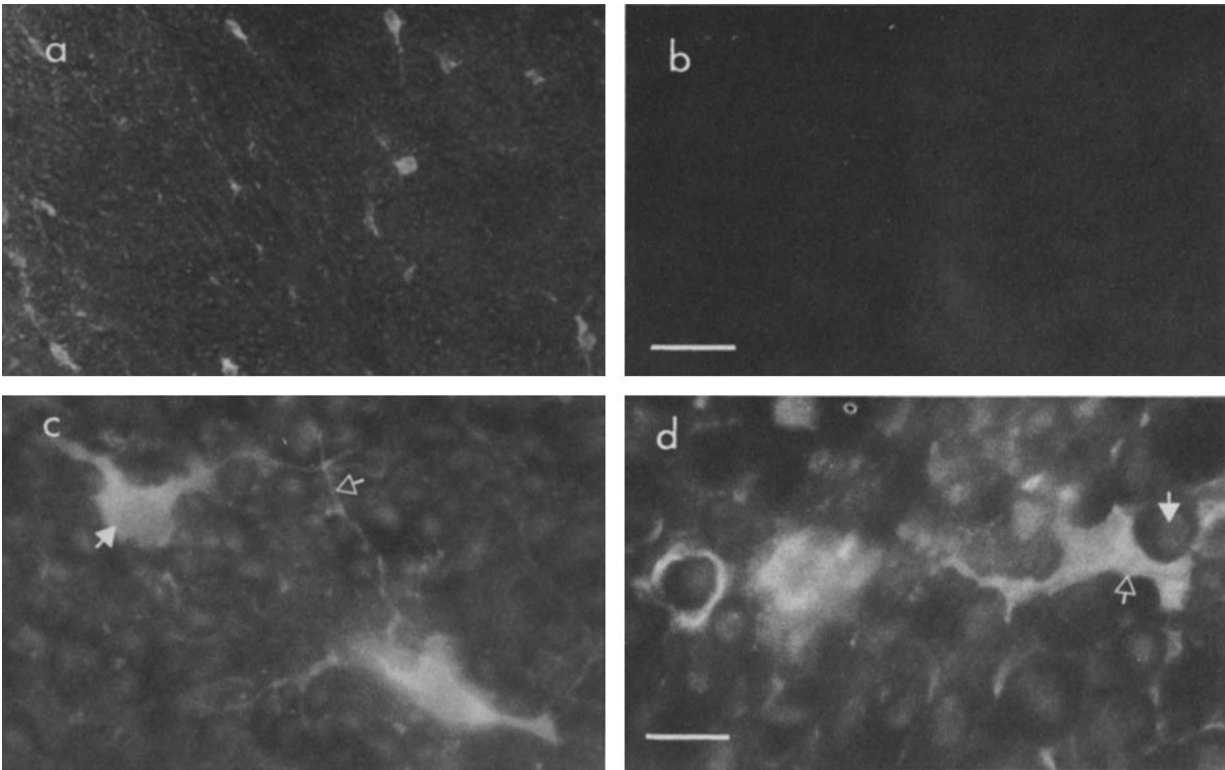
FIGURE 6 Localization of MAP 1A in spinal cord white matter. Cross-sections were cut at the cervical region of the spinal cord. (a) Low magnification view labeled with anti-MAP 1A, illustrating punctate staining of moderate intensity throughout the field, and occasional brightly stained cells. (b) Low magnification view of a control section processed for immunofluorescence microscopy and photographed identically to the section shown in a, except that unconditioned hybridoma medium was used in place of anti-MAP 1A. (c) High magnification view, illustrating cell bodies (solid arrow) and processes (open arrow) of brightly labeled cells identified as oligodendrocytes. Less intense patches of fluorescence throughout the field represent primarily axons cut in approximate cross-sections. (d) High magnification view, in which the cytoplasm of an oligodendrocyte (open arrow) is observed to encircle a myelinated axon. The lightly stained axoplasm (solid arrow) is separated from the oligodendrocyte cytoplasm by the unstained myelin sheath. Bars: (b, for a and b) 50 μm and (d, for c and d) 10 μm. (a and b) × 230. (c and d) × 1,150.

FIGURE 7 Localization of MAP 1A in cerebellum. Sagittal sections are shown at low and high magnification. (A) Low magnification view illustrating the histological layers of the cerebellum: m, molecular layer; g, granule cell layer; wm, white matter; open arrow, Purkinje cell body; closed arrow, Purkinje cell dendrite. Bar, 20 μm. (B) High magnification view of Purkinje cells. Both dendrites (solid arrow) and axons (open arrows) of Purkinje cells contain MAP 1A. (C) High magnification view of white matter. Cellular processes, presumably consisting mostly of axons, were moderately stained throughout this region. Bar, 10 μm. Magnification for B and C was the same. (A) × 250. (B and C) × 750.
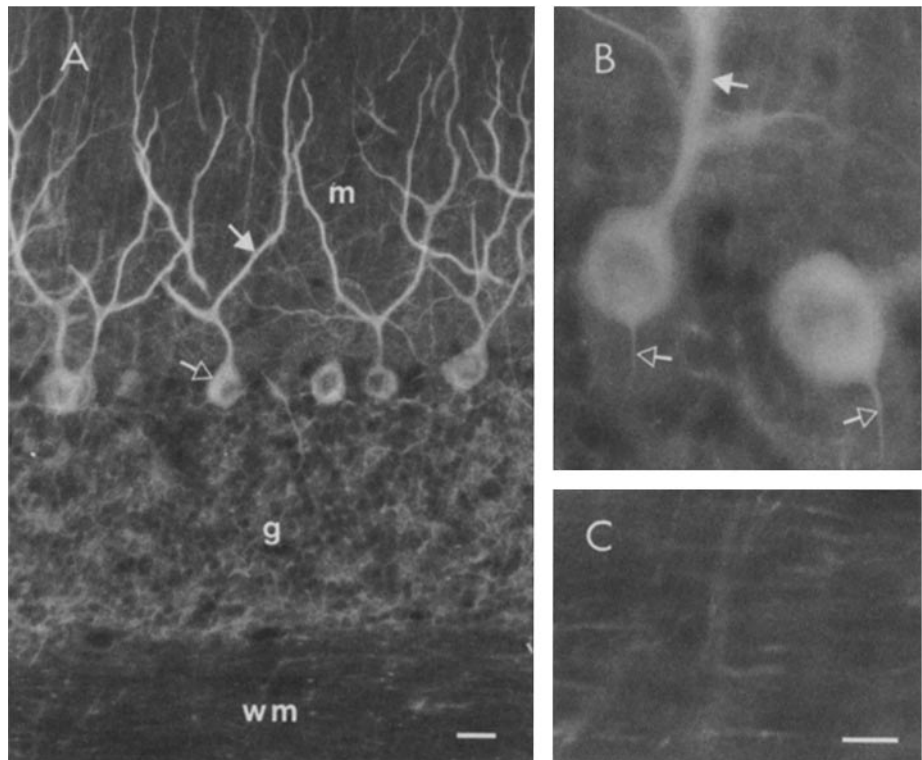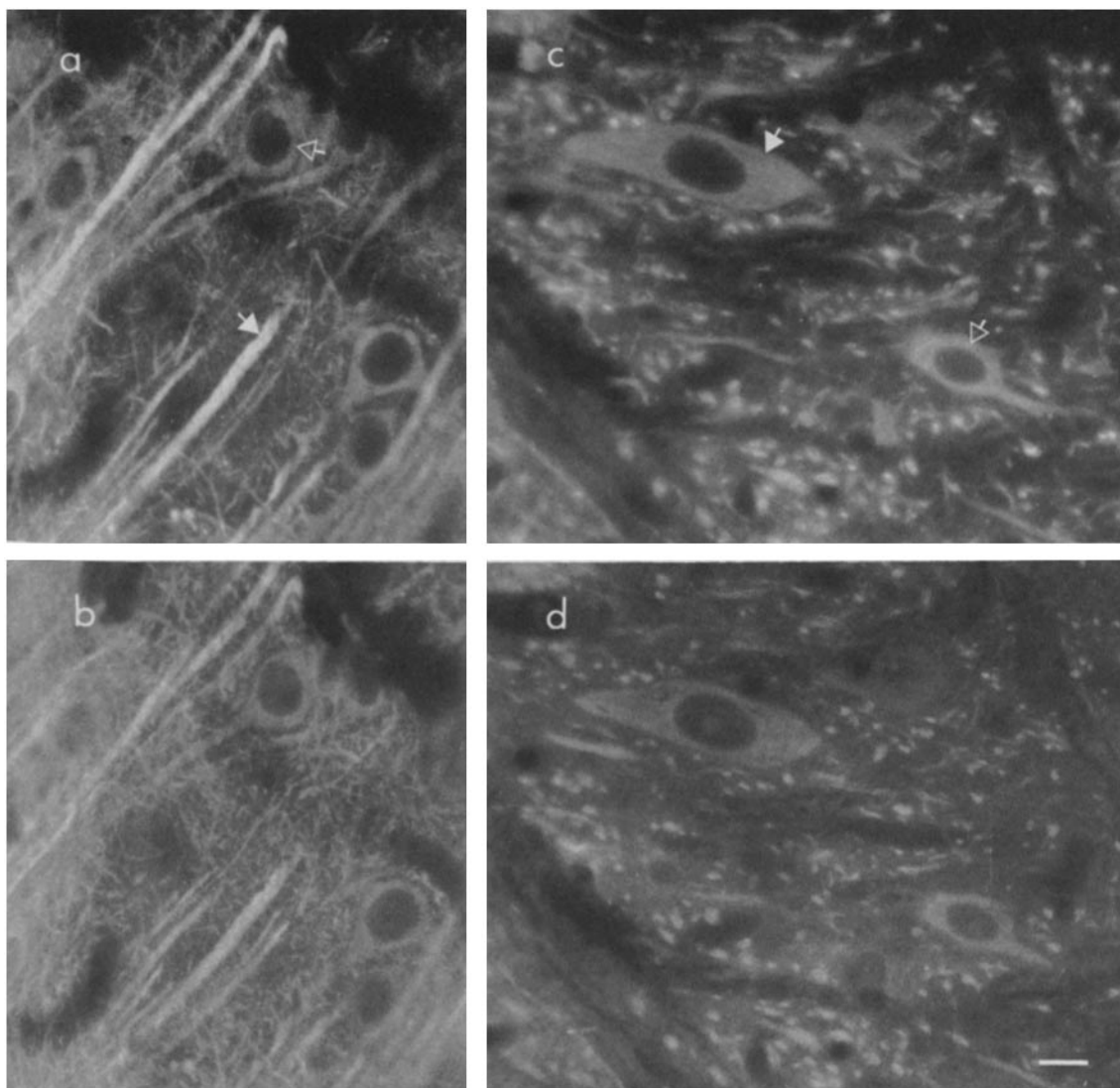
FIGURE 8 Coexistence of MAP 1A and MAP 2 in neurons. Tissue sections were double-labeled with monoclonal anti-MAP 1A and rabbit anti-MAP 2. Second antibodies were rhodamine sheep anti-mouse IgG and fluorescein goat anti-rabbit IgG. (a) Sagittal section of cerebral cortex showing distribution of MAP 1A. Apical dendrites (solid arrow) and perikarya (open arrow) of pyramidal neurons were prominently labeled. (b) Distribution of MAP 2 in the same section. (c) Cross-section of spinal cord gray matter (in mid-cervical region) showing presence of MAP 1A in perikarya of large (solid arrow) and small (open arrow) motor neurons, as well as in numerous processes cut in cross and longitudinal sections. (d) Distribution of MAP 2 in the same section. Bar, 10 μm. × 700.

cord, where motor neurons and numerous processes were stained by anti-MAP 1A and anti-MAP 2, and from the cerebral cortex, where perikarya and apical dendrites of pyramidal neurons were heavily labeled by both antibodies. Other readily identifiable dendritic processes, such as those in the cerebellar cortex, were also stained by both antibodies (not shown). Thus, while MAP 1A alone was detectable in axons and glial cells, both MAP 1A and MAP 2 appeared to be abundant in dendrites and neuronal cell bodies.

Immunofluorescent Detection of MAP 1A in Cultured Brain Cells

To obtain further information on the identity of cells containing MAP 1A, we examined primary cultures of newborn rat brain. Fig. 9 shows cells double-stained with anti-MAP 1A (Fig. 9a) and anti-MAP 2 (Fig. 9b). The latter antibody stained only a limited number of cells in these

cultures, as reported previously (3, 16, 29). Most of these cells were of neuronal morphology and contained receptors for tetanus toxin (3), consistent with the neuronal localization of MAP 2 found in brain and spinal cord (8, 24). In contrast to MAP 2, anti-MAP 1A stained most cells in the culture, albeit with varying intensity. The MAP 2-positive cells of neuronal morphology were consistently stained by anti-MAP 1A, although with only moderate intensity. An additional class of cells, which were MAP 2-negative and were characterized by small cell bodies and long processes, also stained with anti-MAP 1A. These cells appeared to contain the highest concentrations of MAP 1A found in the cultures.

To determine the identity of these cells, we stained the cultures with antibodies specific for glial cells. Fig. 10 shows the result of double immunofluorescence microscopy performed with anti-MAP 1A and antimyelin basic protein, a marker for oligodendrocytes (25). Anti-MBP (Fig. 10b) intensely stained a fraction of the cells in the culture. These
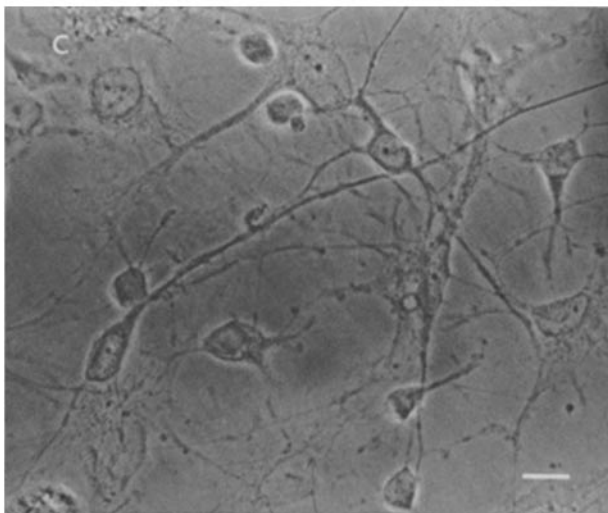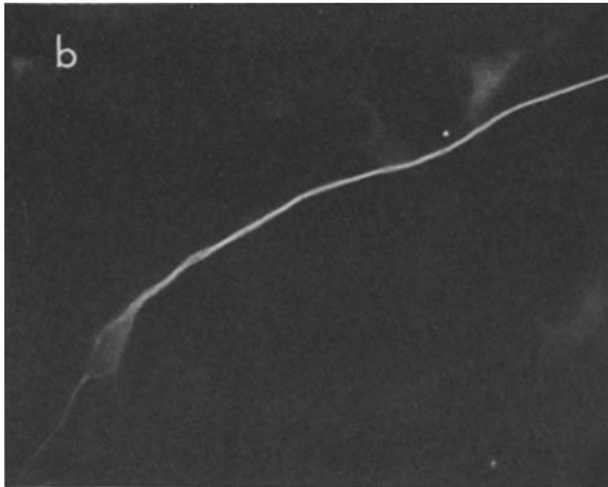
drocytes, consistent with our observations of sections of central nervous system tissue (Fig. 6).

Fig. 11 shows cells double-stained with antibody to glial fibrillary acidic protein, a specific marker for astrocytes, and anti-MAP 1A. Most of the cells in the culture with flat, well-spread morphology stained with anti-GFAP and were judged on this basis to be astrocytes. Those cells also stained positively with anti-MAP 1A, but the intensity of fluorescence was considerably lower than for oligodendrocytes (for a direct comparison see Figs. 9a and 10a). Dividing astrocytes were also observed occasionally, and the mitotic spindles in those cells were brightly stained with anti-MAP 1A. Staining of the spindle was generally observed in dividing cells in these and other cultures, as will be discussed in the accompanying paper (4).

DISCUSSION

Previous work from this laboratory revealed that MAP 1 was as abundant in microtubules purified from bovine white matter as in microtubules purified from cerebral cortex (40). This led us to predict that, in contrast to MAP 2, MAP 1
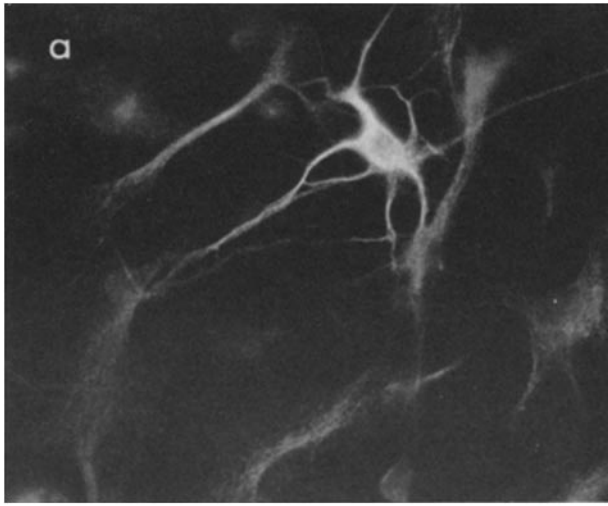

FIGURE 9 Distinct cellular expression of MAP 1A and MAP 2 in primary cultures of newborn rat brain. A coverslip was labeled with monoclonal anti-MAP 1A (a) and rabbit anti-MAP 2 (b), followed by rhodamine sheep anti-mouse IgG and fluorescein goat anti-rabbit IgG. (c) Phase-contrast image of the same field. Bar, 10 μm. × 650.

cells were the same as those most intensely stained with anti-MAP 1A (Fig. 10a). Since the morphology of these cells is characteristic not only of neurons but of oligodendrocytes as well (25), we conclude that MAP 1A is abundant in oligoden-
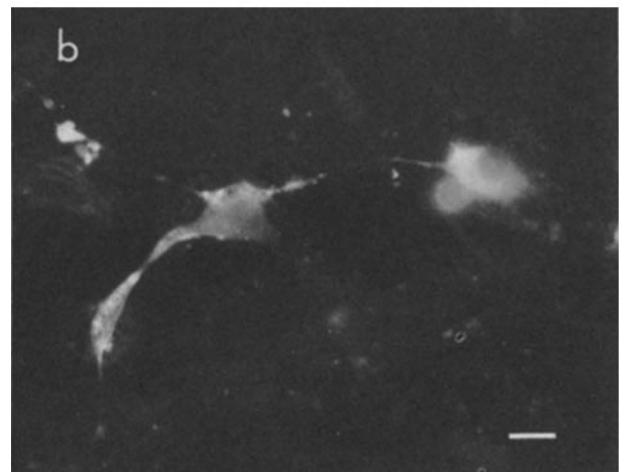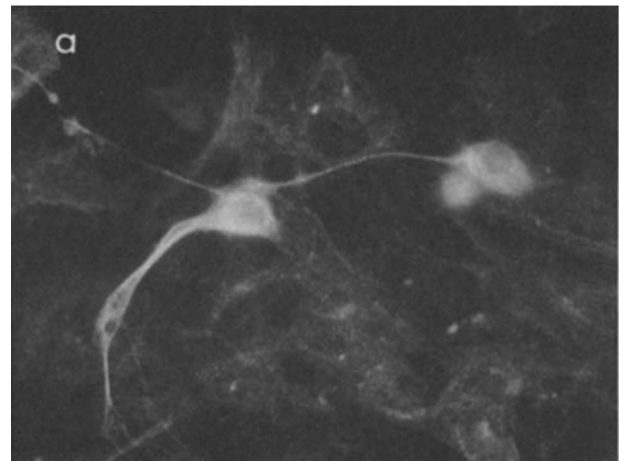
FIGURE 10 Localization of MAP 1A in cultured oligodendrocytes. A coverslip containing primary new born rat brain cells was double labeled with monoclonal anti-MAP 1A (a) and rabbit anti-myelin basic protein (b) followed by rhodamine sheep anti-mouse IgG and fluorescein goat anti-rabbit IgG. Anti-MAP 1A stained two cells in this field very brightly, and produced light staining of numerous other cells. The brightly staining cells also reacted with antimyelin basic protein, identifying them as oligodendrocytes. Bar, 10 μm. × 650.
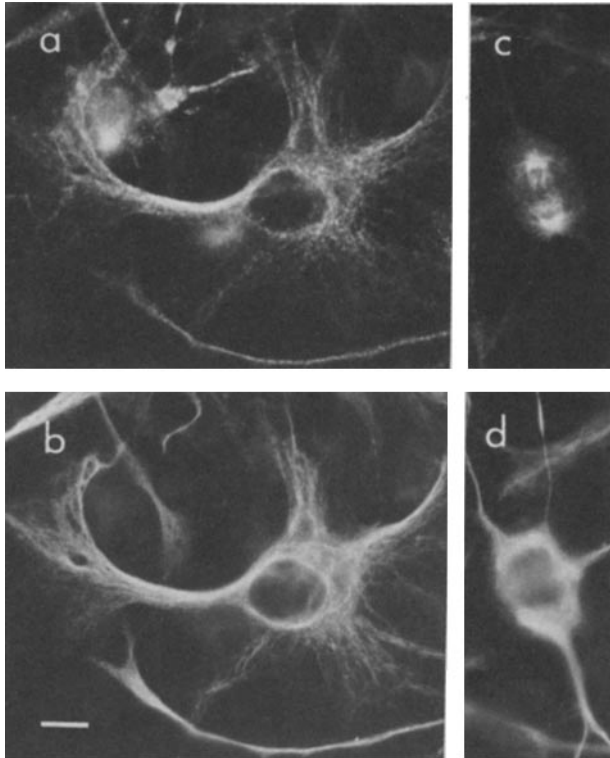
FIGURE 11 Localization of MAP 1A in cultured astrocytes. A coverslip containing primary new born rat brain cells was double labeled with monoclonal anti-MAP 1A (a and c) and rabbit anti-glial fibrillary acidic protein (b and d), a marker for astrocytes. Second antibodies were rhodamine sheep anti-mouse IgG and fluorescein goat anti-rabbit IgG. The large cell shown in a and b is in interphase, while the cell in c and d is undergoing mitosis. Bar, 10 μm. × 650.

would be found in axons, glial cells, or both. In the present study, we examined the distribution of the major polypeptide component of MAP 1 by immunofluorescence microscopy of tissue sections and primary cultures of brain cells using a monoclonal antibody. We have found that MAP 1 consists of at least three electrophoretic species, the principal one of which, designated MAP 1A, is recognized exclusively by our antibody. We found that MAP 1A, like MAP 2, is prominent in neurons. In contrast to MAP 2, MAP 1A was easily detected in rat brain white matter, while both proteins were abundant in gray matter, confirming the earlier biochemical results from our laboratory. We have shown that MAP 1A is present in identifiable axons as well as in identifiable dendrites. In addition, we have found that MAP 1A, unlike MAP 2, is prominent in oligodendrocytes and is detectable in astrocytes, as well. Thus, our work stands in contradiction to that of Matus et al. (23), who concluded on the basis of studies using an antibody preparation directed against unfractionated high molecular weight MAP, that these MAP were absent from axons and glial cells.

Heterogeneity of Map 1

While MAP 1 has been treated as a single biochemical entity, we noted several electrophoretic bands in the MAP 1 region of SDS gels. These bands were more striking in microtubules prepared from white matter than from gray matter (Bloom and Vallee, unpublished observations). We do not know whether this represents a real concentration difference

in MAP 1B and MAP 1C in white matter vs. gray matter, a result that is potentially of considerable interest, or whether MAP 1B and MAP 1C are obscured by the heavy MAP 2 bands in gray matter preparations. Both MAP 1A and MAP 1B ran as relatively broad electrophoretic bands. This was observed both on 4% polyacrylamide gels prepared according to Laemmli (21), as in Fig. 1, and on 3–5% polyacrylamide gradient, SDS-urea gels (not shown) prepared according to Pfister et al. (31). The basis for this behavior is not understood, though it may indicate that MAP 1A and MAP 1B are themselves composed of numerous polypeptides differing slightly in electrophoretic mobility.

The monoclonal antibody we described here reacted with the entire MAP 1A band, indicating that if MAP 1A were complex, the components of this electrophoretic species must be closely related. It is possible that MAP 1B and MAP 1C represent proteolytic fragments of MAP 1A lacking the antigenic site recognized by our antibody. This possibility can be virtually eliminated for MAP 1C because of the resistance of this band to digestion by chymotrypsin (Fig. 1C). If MAP 1C were derived from the larger polypeptides, they should show evidence of a protease resistant region at least as large as MAP 1C. Instead, MAP 1A and MAP 1B were totally digested to products of lower molecular weight than MAP 1C. The question of whether MAP 1B is also a distinct protein or merely a fragment of MAP 1A cannot be answered with certainty at this time. However, in view of the reproducible ratio of the two bands that we have observed in our preparations, and, as noted above, evidence of a specific enrichment of MAP 1B in white matter, we are inclined to consider MAP 1A and MAP 1B distinct proteins. In the following paper (4) we demonstrate by immunofluorescence microscopy that MAP 1A is localized on microtubules in cultured cells, and is therefore a true MAP. At this time we do not know whether MAP 1B and MAP 1C behave as MAP by immunocytochemical criteria. We are currently raising antibodies to MAP 1B and MAP 1C to characterize these species further.

Distribution of MAP 1A in the Nervous System

Several points regarding the distribution of MAP 1A deserve comment. Staining of processes clearly identifiable as axons was observed throughout the brain and spinal cord. However, the staining intensity of axons by anti-MAP 1A was quite variable. In major cerebral white matter tracts (see Figs. 2–4) bright staining was observed, while white matter tracts in the cerebellum and spinal cord (see Fig. 6) tended to be less intensely stained. Climbing fiber and parallel fiber axons of the cerebellar cortex were also weakly stained (see Fig. 7). Thus, we cannot state as a general rule that all axons contain high concentrations of MAP 1A. Perhaps the concentration of MAP 1A varies with axon type, or the antigenic site recognized by our particular antibody is differentially blocked by interaction with other proteins. Alternatively, there may be real differences in the cytoskeletal composition of different axons for reasons that are not yet clear.

The detectability of MAP 1A in axons distinguishes this protein from MAP 2 in terms of subcellular distribution. The discovery of MAP 1A in two types of glial cells further distinguishes this protein from MAP 2. Oligodendrocytes were brightly stained in culture, and cells with the characteristics of oligodendrocytes were prominently labeled in spinal cord white matter (Fig. 6) and other white matter regions. Astrocytes were also positive for MAP 1A in primary brain cell

cultures, though we were unable to detect MAP 1A in these cells in vivo. Several factors may explain why astrocytes were stained by anti-MAP 1A in culture, but not in vivo. First, immunofluorescence microscopy is more sensitive for cultured cell monolayers than for the tissue sections, due in part to differences in specimen thickness (no more than a few μm for cultures vs. 20 μm for tissue sections). Next, the staining intensity with anti-MAP 1A was substantially greater for dividing than nondividing astrocytes in culture. Since mature brain and spinal cord contain few, if any, dividing astrocytes, it is not surprising that no such cells were stained by anti-MAP 1A in tissue sections. Finally, immature astrocytes, such as those present in the primary newborn rat brain cultures, have been reported to be far richer in microtubules than more mature astrocytes, such as those found in the 3–4-mo-old rats from which we obtained brain sections (30). Collectively, these considerations may explain why astrocytes were stained by anti-MAP 1A in primary brain cell cultures, but not in tissue sections.

The distributions of MAP 1A and MAP 2 in the nervous system are clearly distinct. However, similarities in their distributions are noteworthy. Throughout brain and spinal cord gray matter, both MAP 1A and MAP 2 are heavily concentrated in neuronal dendrites and cell bodies. Double immunofluorescence with antibodies to the two MAP have enabled us to demonstrate that individual cell bodies and dendrites may contain high levels of both MAP 1A and MAP 2. Examples of this in the cerebral cortex and spinal cord are shown in Fig. 8. Thus, while axons, oligodendrocytes and astrocytes express detectable levels of MAP 1A only, this protein may be found in combination with high levels of MAP 2 in neuronal perikarya and dendrites.

Functional Considerations

Two observations regarding the distribution of MAP 1A and MAP 2 are relevant to the question of how these MAP function in cells. First, the distribution of the two proteins is not identical. This conclusion was suggested by our earlier biochemical results (40), but can now be made more emphatically on the basis of immunofluorescence microscopy. With regard to this point, we note that differences between MAP 1 and MAP 2, such as those observed in axons and in glial cells, may be relative rather than absolute. This is suggested by our earlier observation that MAP 2 was not entirely absent in white matter, but was present at low levels (40), despite the absence of significant immunoreactivity in white matter (8, 24; Fig. 3b in this paper). Nevertheless, whether the absence of MAP 2 in cells containing MAP 1A is absolute or relative, it seems reasonable to conclude now that the two proteins cannot function cooperatively in cells in a simple stoichiometric fashion. Though MAP 1 and MAP 2 are immunologically cross-reactive, respectively, to the erythrocyte membrane proteins ankyrin and spectrin (2, 7), which interact with one another in a defined stoichiometry, our studies indicate that MAP 1A and MAP 2 are likely to act independently in the cell. The second observation relevant to the question of MAP function is the coincident presence of MAP 1A and MAP 2 within individual neuronal perikarya and dendritic processes (Fig. 8). Here, the two proteins may serve different functions within the same cytoplasm.

Existing evidence from this laboratory indicates that MAP 2 is involved at least in mediating the interaction of microtubules with intermediate filaments (3). In the accompanying

paper (4) we report that no evidence supporting such a function for MAP 1A was obtained. Rather, this protein occurs in the mitotic spindle of a wide variety of cells, as well as on cytoplasmic microtubules in nondividing cells. The functional implications of these findings and further observations are discussed in the next paper (4).

REFERENCES

1. Aamodt, E., and R. C. Williams, Jr. 1983. MAPs mediate association of microtubules and neurofilaments in vitro. *Biophys. J.* 41:86a. (Abstr.)
2. Bennett, V., and J. Davis. 1981. Erythrocyte ankyrin: immunoreactive analogues are associated with mitotic structures in cultured cells and with microtubules in brain. *Proc. Natl. Acad. Sci. USA.* 78:7550–7554.
3. Bloom, G. S., and R. B. Vallee. 1983. Association of microtubule-associated protein 2 (MAP 2) with microtubules and intermediate filaments in cultured brain cells. *J. Cell Biol.* 96:1523–1531.
4. Bloom, G. S., F. C. Luca, and R. B. Vallee. 1984. Widespread cellular distribution of MAP 1A in the mitotic spindle and on interphase microtubules. *J. Cell Biol.* 98:331–340.
5. Borisy, G. G., J. M. Marcum, J. B. Olmsted, D. B. Murphy, and K. A. Johnson. 1975. Purification of tubulin and associated high molecular weight proteins from porcine brain and characterization of microtubule assembly *in vitro. Ann. N.Y. Acad. Sci.* 253:107–132.
6. Bulinski, J. C., and G. G. Borisy. 1979. Self-assembly of microtubules in extracts of cultured HeLa cells and the identification of HeLa microtubule-associated proteins. *Proc. Natl. Acad. Sci. USA.* 76:293–297.
7. Davis, J., and V. Bennett. 1982. Microtubule-associated protein 2, a microtubule-associated protein from brain, is immunologically related to the α subunit of erythrocyte spectrin. *J. Biol. Chem.* 257:5816–5820.
8. De Camilli, P., P. Miller, F. Navone, W. Theurkauf, and R. B. Vallee. 1984. Patterns of MAP 2 distribution in the nervous system studied by immunofluorescence. *Neuroscience.* In press.
9. Forni, L. 1979. Reagents for immunofluorescence and their use for studying lymphoid cell products. *In* Immunological Methods. I. Lefkovits and B. Pernis, editors. Academic Press, Inc., New York. 151–166.
10. Goldman, M. 1968. Fluorescent Antibody Methods. Academic Press, Inc., New York. 150.
11. Greene, L. A., R. K. H. Liem, and M. L. Shelanski. 1983. Regulation of a high molecular weight microtubule-associated protein in PC12 cells by nerve growth factor. *J. Cell Biol.* 96:76–83.
12. Griffith, L., and T. D. Pollard. 1978. Evidence for actin filament-microtubule interaction mediated by microtubule-associated proteins. *J. Cell Biol.* 78:958–965.
13. Griffith, L., and T. D. Pollard. 1982. The interaction of actin filaments with microtubules and microtubule-associated proteins. *J. Biol. Chem.* 257:9143–9151.
14. Herzog, W., and K. Weber. 1978. Fractionation of brain microtubule-associated proteins. *Eur. J. Biochem.* 92:1–8.
15. Hirokawa, N. 1982. Cross-linker system between neurofilaments, microtubules, and membranous organelles in frog axons revealed by quick-freeze, deep-etching method. *J. Cell Biol.* 94:129–142.
16. Izant, J. G., and J. R. McIntosh. 1980. Microtubule-associated proteins: a monoclonal antibody to MAP 2 binds to differentiated neurons. *Proc. Natl. Acad. Sci. USA.* 77:4741–4745.
17. Kim, H., L. I. Binder, and J. L. Rosenbaum. 1979. The periodic association of MAP 2 with brain microtubules in vitro. *J. Cell Biol.* 80:266–276.
18. Kohler, G., and C. Milstein. 1976. Derivation of specific antibody-producing tissue culture and tumor lines by cell fusion. *Eur. J. Immunol.* 6:511–519.
19. König, J. F. R., and R. A. Klippel. 1970. The Rat Brain: A Stereotoxic Atlas of the Forebrain and Lower Parts of the Brain Stem. Robert E. Krieger Publishing Co., Huntington, NY.
20. Kuznetsov, S. A., V. I. Rodionov, V. I. Gelfand, and V. A. Rosenblat. 1982. Microtubule-associated protein MAP 1 promotes microtubule assembly *in vitro. FEBS (Fed. Eur. Biochem. Soc.) Lett.* 135:241–244.
21. Laemmli, U. K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.
22. Le Terrier, J. F., R. Liem, and M. L. Shelanski. 1982. Interactions between neurofilaments and microtubule-associated proteins: a possible mechanism for interorganellar binding. *J. Cell Biol.* 95:982–986.
23. Matus, A., R. Bernhardt, and T. Hugh-Jones. 1980. High molecular weight microtubule-associated proteins are preferentially associated with dendritic microtubules in brain. *Proc. Natl. Acad. Sci. USA.* 78:3010–3014.
24. Miller, P., U. Walter, W. E. Theurkauf, R. B. Vallee, and P. De Camilli. 1982. Frozen tissue sections as an experimental system to reveal specific binding sites for the regulatory subunit of type II cAMP-dependent protein kinase in neurons. *Proc. Natl. Acad. Sci. USA.* 79:5562–5566.
25. Mirsky, R., J. Winter, E. R. Abney, R. M. Pruss, J. Gavrilovic, and M. C. Raff. Myelin-specific proteins and glycolipids in rat Schwann cells and oligodendrocytes in culture. *J. Cell Biol.* 84:483–494.

26. Murphy, D. B., and G. G. Borisy. 1975. Association of high-molecular-weight proteins with MT's and their role in MT assembly *in vitro*. *Proc. Natl. Acad. Sci. USA.* 72:2696–2700.
27. Olmsted, J. B., and H. D. Lyon. 1981. A microtubule-associated protein specific to differentiated neuroblastoma cells. *J. Biol. Chem.* 256:3507–3511.
28. Ouchterlony, O. 1948. *Ark. Minerol. Geol.* B26:No. 14,1.
29. Peloquin, J. G., and G. G. Borisy. 1979. Cell and tissue distribution of the major high molecular weight microtubule-associated protein from brain. *J. Cell Biol.* 83(2, Pt. 2):338a. (Abstr.)
30. Peters, A., S. L. Palay, and H. DeF. Webster. 1976. The Fine Structure of the Nervous System. W. B. Saunders Co., Phiadelphia.
31. Pfister, K. K., R. B. Fay, and G. B. Witman. 1982. Purification and polypeptide composition of dynein ATPases from *Chlamydomonas* flagella. *Cell Motility.* 2:525–547.
32. Sattilaro, R. F., W. L. Dentler, and E. L. LeCluyse. 1981. Microtubule-associated proteins (MAPs) and the organization of actin filaments in vitro. *J. Cell Biol.* 90:467–473.
33. Sherline, P., Y. C. Lee, and L. S. Jacobs. 1977. Binding of microtubules to pituitary secretory granules and secretory granule membranes. *J. Cell Biol.* 72:380–389.
34. Sloboda, R. D., S. A. Rudolph, J. L. Rosenbaum, and P. Greengard. 1975. Cyclic AMP-dependent endogenous phosphorylation of a microtubule-associated protein. *Proc. Natl. Acad. Sci. USA.* 72:177–181.
35. Suprenant, K. A., and W. L. Dentler. 1982. Association between endocrine pancreatic secretory vesicles and in vitro assembled microtubules is dependent upon microtubule-associated proteins. *J. Cell Biol.* 93:164–174.
36. Theurkauf, W. E., and R. B. Vallee. 1982. Molecular characterization of the cAMP-dependent protein kinase bound to microtubule-associated protein 2. *J. Biol. Chem.* 257:3284–3290.
37. Theurkauf, W. E., and R. B. Vallee. 1983. Extensive cAMP-dependent and cAMP-independent phosphorylation of microtubule-associated protein 2. *J. Biol. Chem.* 258:7883–7886.
38. Valdivia, M. M., J. Avila, J. Coll, C. Colaco, and I. V. Sandoval. 1982. Quantitation and characterization of the microtubule-associated MAP 2 in porcine tissues and its isolation from porcine (PK15) and human (HeLa) cell lines. *Biochem. Biophys. Res. Commun.* 105:1241–1249.
39. Vallee, R. B. 1980. Structure and phosphorylation of microtubule-associated protein 2 (MAP 2). *Proc. Natl. Acad. Sci. USA.* 77:3206–3210.
40. Vallee, R. B. 1982. A taxol-dependent procedure for the isolation of microtubules and microtubule-associated proteins. *J. Cell Biol.* 92:435–442.
41. Vallee, R. B., and G. G. Borisy. 1977. Removal of the projections from cytoplasmic microtubules *in vitro* by digestion with trypsin. *J. Biol. Chem.* 252:377–382.
42. Vallee, R. B., M. J. Dibartolomeis, and W. E. Theurkauf. 1981. A protein kinase bound to the projection portion of MAP 2 (microtubule-associated protein 2). *J. Cell Biol.* 90:568–576.
43. Vallee, R. B., and S. Davis. 1983. Low molecular weight microtubule-associated proteins are light chains of microtubule-associated protein 1 (MAP 1). *Proc. Natl. Acad. Sci. USA.* 80:1342–1346.
44. Vallee, R. B., and G. S. Bloom. 1984. High molecular weight microtubule-associated proteins. *In* Modern Cell Biology. B. H. Satir, Series Editor. Alan R. Liss, Inc., New York. Vol. 3. In press.
45. Weatherbee, J. A., R. B. Luftig, and R. R. Weihing. 1980. Purification and reconstitution of HeLa cell microtubules. *Biochemistry.* 19:4116–4123.
46. Weingarten, M., A. Lockwood, S. Hwo, and M. Kirschner. 1975. A protein factor essential for microtubule assembly. *Proc. Natl. Acad. Sci. USA.* 72:1858–1862.