Immunocytochemical Localization of Microtubule-associated Protein 1 in Rat Cerebellum Using Monoclonal Antibodies

GERDA HUBER and ANDREW MATUS
Friedrich Miescher-Institut, CH-4002 Basel, Switzerland

ABSTRACT Immunohistochemical staining with monoclonal antibodies showed that microtubule-associated protein 1 (MAP1) has a restricted cellular distribution in the rat cerebellum. Anti-MAP1 staining was found only in neurons, where it was much stronger in dendrites than in axons. There were striking variations in the apparent concentration of MAP1 in different classes of neurons. Purkinje cells were the most strongly labeled, while granule cell neurons gave a faint, threshold-level reaction with the antibody. The reaction of Golgi neurons was intermediate between these two extremes. Equivalent results were obtained using two different methods of tissue preparation. Thus MAP1 appears to be a neuron-specific protein that is highly concentrated in dendrites and occurs at markedly different levels in different types of neurons. These observations provide further indications of heterogeneity among brain microtubules.

Microtubule-associated protein 1 (MAP1)[1] is a high molecular weight ($M_r > 300,000$) polypeptide that co-purifies with brain microtubules through several cycles of assembly (3, 8, 27), and is one of the most prominent components of brain-derived microtubules. Methods for its purification have only recently been introduced (18, 29) so that its properties are not as yet well characterized. Chemically, MAP1 differs from the other high molecular weight brain microtubule protein, MAP2, by peptide mapping (18) and by being heat labile whereas MAP2 is heat stable (11). In terms of function, there are now two reports that MAP1 is effective in promoting the assembly of tubulin into microtubules (19, 29). It has also been reported that synthesis of MAP1 is selectively enhanced in pheochromocytoma cells when they have been induced to grow neurites by treatment with nerve growth factor (12).

Almost nothing is known regarding the cellular affiliation of MAP1 although recent studies have indicated that it is absent from non-neural cells, which contain MAPs of different apparent molecular weights on SDS gels than those found in brain microtubules (1, 4, 5, 9, 30). To date, the only information on the cellular distribution of MAP1 in brain is that it occurs in taxol-induced precipitates from both gray and white matter (28). Our interest in high molecular weight MAPs was stimulated by an earlier study in which we found that polyclonal antisera against certain high M_r MAPs selectively stained neuronal dendrites (2, 20). We have subsequently begun to raise monoclonal antibodies against MAPs to get a better resolution of their tissue distribution and cellular localization. In the present study we used monoclonal antibodies against MAP1 to determine its distribution in rat cerebellum. The results show that MAP1 is neuron-specific, is more concentrated in dendrites than axons, and is more concentrated in some types of neurons than in others.

MATERIALS AND METHODS

Mice were immunized with MAPs isolated from twice-recycled rat brain microtubules (17). Spleen cell-myeloma hybridomas were formed and grown as previously described (14), except that the fusion products were cultured in methyl cellulose to facilitate the recovery of single viable colonies (7). Hybridoma media were screened first against dots of microtubule protein on nitrocellulose squares (13) and then, if positive, against SDS gel blots to identify the antigen being recognized (22). Three independently generated monoclonals secreting antibodies against MAP1 were recovered. Their monoclonal nature was established by subcloning at limiting dilution. Chain typing showed that all three antibodies are IgG1. Each gave the same results in all the immunological procedures used, the only detectable difference being in the titer of antibody secreted by clones derived from the different primary hybridoma lines. Monoclonal antibodies against MAP2 were obtained from cloned hybridomas recovered from the same fusion (21).

Rat brain tissue was prepared for immunoperoxidase staining in either of two ways. In most experiments rat brains were fixed by transcardiac perfusion of nembutal-anesthetized animals after injection of heparin-saline into the left ventricle and briefly flushing out the blood with isotonic saline. The fixative was 200 ml of 4% paraformaldehyde containing 0.5% glutaraldehyde in 50 mM phosphate buffer, pH 7.4. The tissue was sectioned at 40 μM in a Vibratome (Kontron Instruments, Zürich). Alternatively, rats were guillotined, and a small piece of cerebellum was rapidly removed and immediately plunged into isopentane cooled in liquid nitrogen. The frozen tissue was sectioned at 20 μm in a cryostat and fixed for 5 min in acetone at −20°C. From the initial freezing until the end of fixation the tissue was not allowed to thaw. The fixed sections were air dried and washed three times with PBS before being stained with anti-MAP1. Immunohistochemical staining was performed as previously

[1] *Abbreviation used in this paper:* MAP1, microtubule-associated protein 1.

described (23) except that 4-chloro-1-naphthol was used as chromogen. The source of antibody used in these experiments was supernatant medium from the cloned hybridomas.

RESULTS

MAP1 is identified by its co-purification with brain microtubules through repeated cycles of assembly/disassembly and its characteristic position as the slowest migrating polypeptide during SDS gel electrophoresis of microtubule proteins (3, 8, 24). Fig. 1 shows that our monoclonal antibodies react selectively with this band. In addition we have shown that this same antigen-bearing polypeptide conforms to MAP1 in being heat-labile (11). The anti-MAP1 antibodies do not cross-react with MAP2 or any other microtubular protein, including the low molecular weight MAPs that have recently been reported to form a complex with MAP1 (29). By immunoassay, MAP1 is not detectable in nonmicrotubular brain subcellular fractions including brain intermediate filaments, brain actomyosin, and synaptosomes (15).

When monoclonal antibodies against MAP1 were used to stain sections of rat cerebellum, the reaction was restricted to neurons (Fig. 2). Glial cells and their processes were never labeled, either in aldehyde-fixed tissue (Fig. 2) or in tissue fixed with acetone (Fig. 3). A neuronal localization of MAP1 is also supported by immunoassay of homogenates of non-neural tissues (liver, kidney, spleen, and muscle) none of which gave a detectable reaction for MAP1, whereas brain homogenates were clearly positive (15). Similarly, sections of non-neural tissues showed no detectable immunohistochemical staining by anti-MAP1.

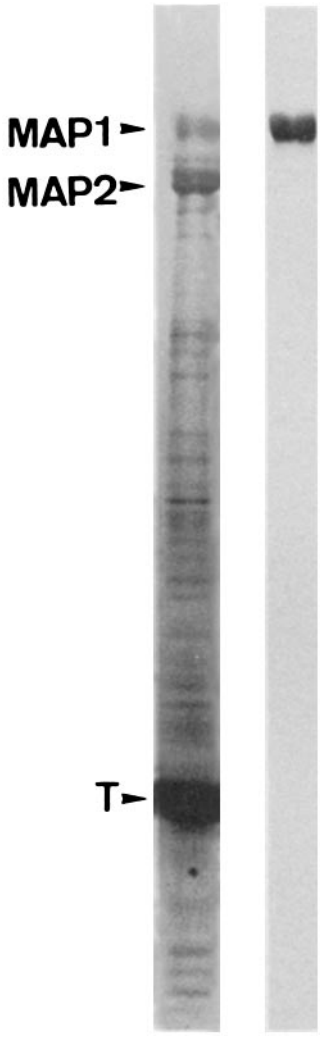

FIGURE 1 Nitrocellulose blots of SDS-polyacrylamide gels in which microtubule proteins had been separated. The left-hand strip is stained with amido black for protein, the right-hand strip with monoclonal antibody. The positions of MAP1, MAP2, and tubulin (*T*) are indicated.

Two kinds of controls indicate the specificity of the anti-MAP1 staining. First, brain sections of the types used here are not stained by the supernatant medium of the myeloma cells used to form the hybrids. Second, in surveying several hundred monoclonal antibodies against neural antigens (14), a wide variety of staining patterns was encountered but only the three primary hybridoma lines secreting anti-MAP1 gave the pattern described here.

Anti-MAP1 staining was unequally distributed between the major histological divisions of the cerebellar cortex. The stronger staining was found in the molecular layer, where it was associated with Purkinje cells and their dendrites (Fig. 2*a*). Staining was much weaker in the granule cell layer. Axons in the white matter were very faintly, although distinctly and reproducibly, stained, and this was more clearly seen in acetone-fixed material (Fig. 3, *a*, *c*, and *e*).

The most striking feature of the anti-MAP1 staining was the large variation among different neuronal cell types. The intense labeling of the Purkinje cells represents one extreme, and the faint, threshold-level staining of the granule cell neurons the other (Figs. 2*b* and 3*b*). Intermediate between these two was the moderate staining of Golgi cell neurons in the granular layer (Fig. 2*a*). This variation of antigen levels in these different cell types was also apparent when we examined sections stained with increasing dilutions of the antibody (Fig. 2, *a–c*). At moderate dilutions the granule cell neuron staining was reduced to threshold levels whereas the Purkinje cell and Golgi neuron staining was not diminished (Fig. 2*b*). At higher dilutions the granule cell staining disappeared, the Golgi neuron staining was markedly weaker, and the Purkinje cell staining showed the first signs of lessening (Fig. 2*c*).

We considered the possibility that the anti-MAP1 staining pattern might simply reflect the relative sizes of different types of neurons and their processes or the differing concentrations of microtubules within them. However, staining sections with monoclonal antibodies against MAP2 (15, 21) showed that this was not the case: anti-MAP2 stains granule cells more strongly then Purkinje cells and does not stain white matter at all (compare Fig. 2, *c* and *d*). (The thin varicose processes that are stained by anti-MAP2 appear to be dendrites of interneurons; R. Bernhardt and A. Matus, submitted for publication.)

To ensure that these intracellular differences were independent of the method of tissue preparation, we also stained sections of cerebellum fixed under entirely different circumstances. In these cases a small fragment of fresh tissue, rapidly dissected from a newly killed rat, was rapidly frozen, cryostat sectioned, and acetone fixed without being allowed to thaw. The anti-MAP1 staining pattern of tissue prepared this way was essentially the same as for tissue fixed by aldehyde perfusion (Fig. 3), with only neurons being labeled and the same variation in staining intensity between Purkinje, Golgi, and granule cells. As before, the reaction in dendrites was much stronger than that in axons (Fig. 3*a*) including MAP1 reactivity in the initial axon segment of Purkinje cells (Fig. 3*b*). Fig. 3*c* shows the distinct axonal staining in the white matter with anti-MAP1. By way of comparison, a section from the same tissue block stained with anti-MAP2 is shown in Fig. 3*d*. Note that in this case there is no detectable axonal staining.

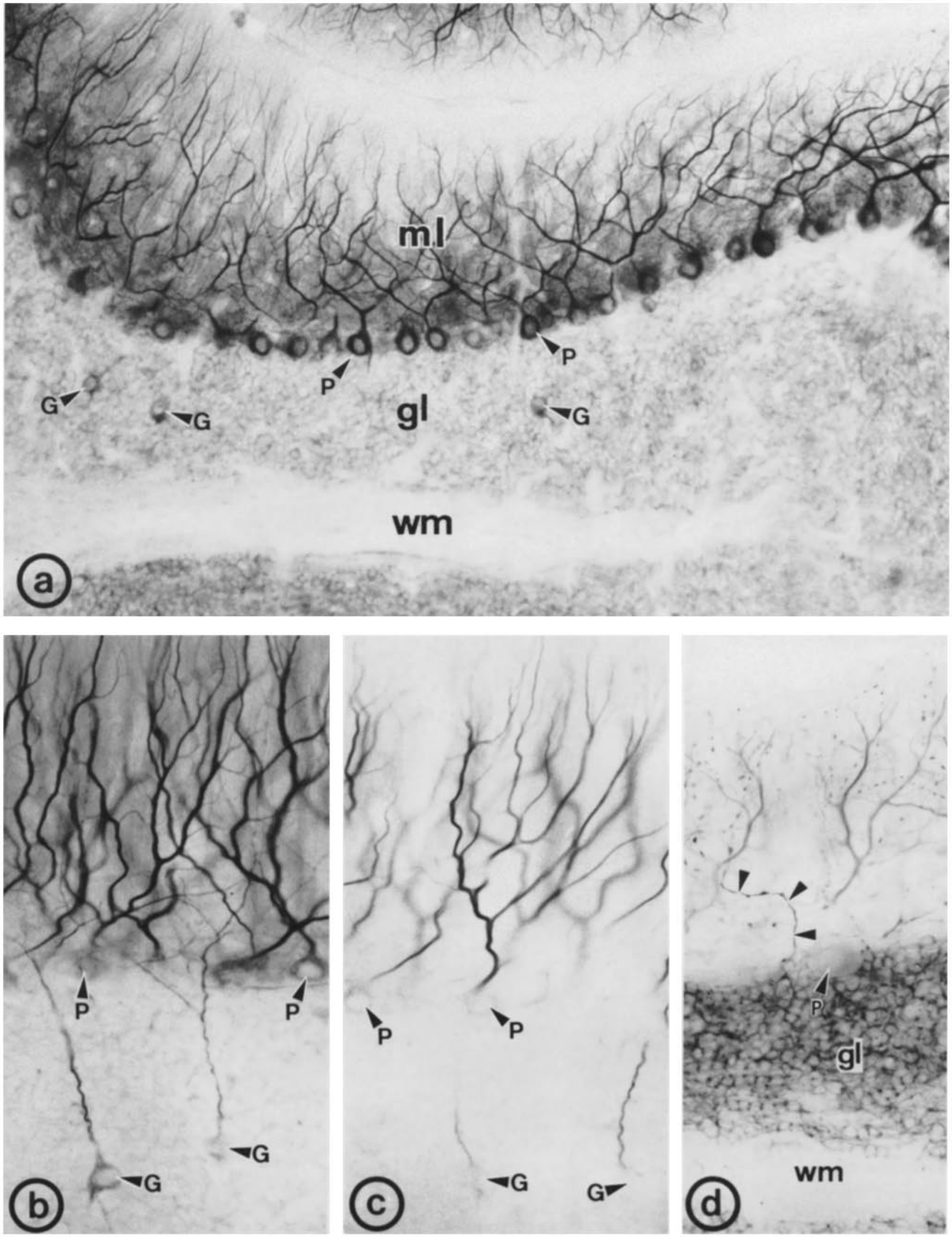

FIGURE 2 Sections of rat cerebellar tissue, perfused fixed with aldehydes, immunoperoxidase-stained with various dilutions of anti-MAP1 (*a–c*) or anti-MAP2 (*d*). All the MAP1-stained sections are taken from the same tissue block. The same results were obtained with sections from other brains. (*a*) Undiluted antibody. The major tissue divisions are identified; *ml*, molecular layer; *gl*, granule cell layer; *wm*, white matter. A few Purkinje cell bodies (*P*) and Golgi cells (*G*) are indicated. (*b* and *c*) Sections stained with increasing dilutions of anti-MAP1: *b*, 1:25; *c*, 1:100. (*d*) Section stained with anti-MAP2 at 1:100 dilution. The labeling is the same as in *a*. In *d* the unmarked arrowheads indicate fine varicose fibers not stained by anti-MAP1. (*a*) × 200; (*b–d*) × 300.

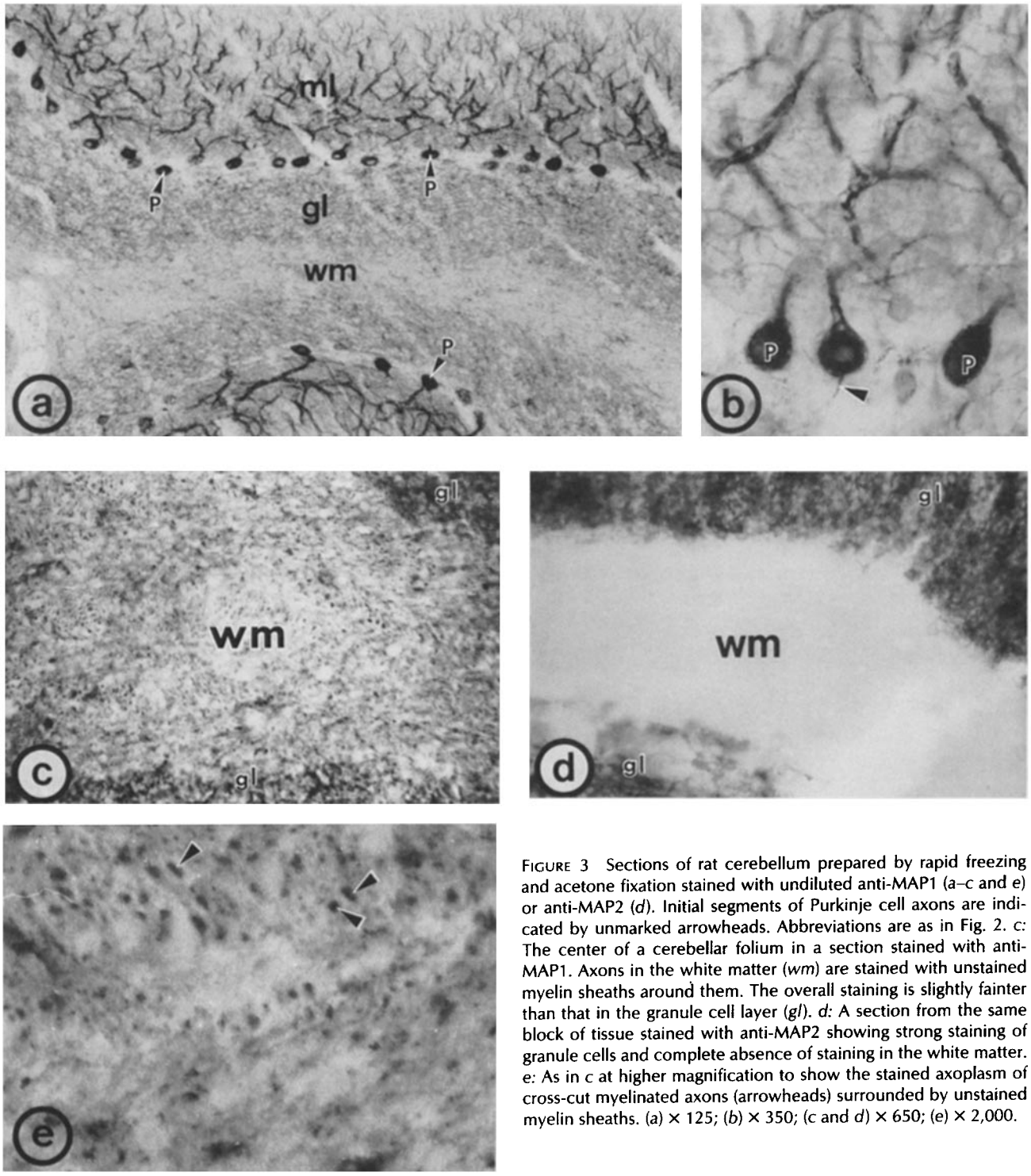

FIGURE 3 Sections of rat cerebellum prepared by rapid freezing and acetone fixation stained with undiluted anti-MAP1 (a–c and e) or anti-MAP2 (*d*). Initial segments of Purkinje cell axons are indicated by unmarked arrowheads. Abbreviations are as in Fig. 2. *c:* The center of a cerebellar folium in a section stained with anti-MAP1. Axons in the white matter (*wm*) are stained with unstained myelin sheaths around them. The overall staining is slightly fainter than that in the granule cell layer (*gl*). *d:* A section from the same block of tissue stained with anti-MAP2 showing strong staining of granule cells and complete absence of staining in the white matter. *e:* As in *c* at higher magnification to show the stained axoplasm of cross-cut myelinated axons (arrowheads) surrounded by unstained myelin sheaths. (*a*) × 125; (*b*) × 350; (*c* and *d*) × 650; (e) × 2,000.

It is also worth noting that although both anti-MAP1 and anti-MAP2 stain granule cell perikarya, there is very little if any reaction of either antibody with the parallel fiber axons that emerge from these cells.

DISCUSSION

The observations reported here indicate that in the cerebellum, MAP1, one of the major proteins associated with brain microtubules, is exclusively associated with neurons, is much more concentrated in dendrites than axons, and is present at widely different levels in various neuron types. This conclusion is based on immunoperoxidase staining of both tissue prepared by perfusion with cross-linking aldehyde fixatives and tissue rapidly frozen and fixed with acetone, a protein denaturant. In both cases, only nerve cells were stained. In addition, immunoassay showed that MAP1, which is readily detectable in brain homogenates, is not detectable in non-neural tissues. The neuronal specificity of MAP1 adds to a growing body of evidence indicating that different cell types possess various characteristic MAPs (1, 4–6, 9, 10, 26, 30).

In earlier studies using polyclonal antibodies we showed

that certain high molecular weight MAPs were neuron-specific and limited to dendrites (2, 20). We have since determined that our anti-high-molecular-weight-MAP sera are directed against MAP2 (21), and a specific association of MAP2 with neurons was also indicated by an independent study with monoclonal antibodies (16). Thus, MAP1 represents a second neuron-specific, dendrite-concentrated microtubule protein. However, unlike MAP1, MAP2 is present at high concentrations in all categories of cerebellar neurons and is exclusively limited to dendrites, as shown by immunostaining with both polyclonal antisera (2, 20) and monoclonal antibodies against MAP2 (21). It is also worth noting that we have found this pattern of neuronal and dendritic localization only for MAP1 and MAP2, while other monoclonal antibodies have revealed novel MAPs that are associated with glia and axons (21).

A further novel feature of our results is the finding that various classes of neurons in the cerebellum contain different concentrations of MAP1. However, all cerebellar neurons contain microtubules (25) and are stained throughout their cytoplasm by antitubulin (20). Thus the anti-MAP1 staining patterns suggest that in the cerebellum, microtubule heterogeneity with respect to MAP content exists between neurons and glia, between different types of neurons, and between dendrites and axons. Recent data suggest that MAP1 may be a regulator of microtubule assembly (19) and hence of neurite growth (12). If this is so, then its differential distribution within individual neurons and among various types of neurons would appear to be of considerable potential significance in cerebellar development.

Received for publication 19 April 1983, and in revised form 20 October 1983.

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