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Differential distribution of β-tubulin isotypes in cerebellum

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We describe the structure and expression of a mammalian β-tubulin isotype (Mβ6) that is weakly expressed in testis but is abundant in developing brain, with transcripts declining to lower levels in the adult brain. The expression of Mβ6 was undetectable in any other mouse tissue examined. A serum specific for this isotype was prepared using a cloned fusion protein as immunogen. Mβ6 is one of five known β-tubulin isotypes expressed in brain, and using the anti-Mβ6 serum along with sera, anti-Mβ2, anti-Mβ3/4 and anti-Mβ5, previously characterized, we have examined the pattern of expression of β-tubulin isotypes in rat cerebellum. The isotypes each have characteristic cell-type specific patterns of localization in cerebellum. Mβ2, Mβ3/4 and Mβ5 are present in both neuronal and non-neuronal cells, but in contrast Mβ6 was only detectable in neurons in tissue sections and in dissociated cerebellar cell culture. The majority of sequence differences among the β-tubulin isotypes lie at the carboxy terminus, the region of β-tubulin involved in MAP binding. In the case of Mβ2 and Mβ6, the patterns of expression are similar or identical to the patterns of expression of MAP3 and MAP1A respectively. These results suggest that β-tubulin isotypes may contribute to the determination of the specific association of MAPs with microtubules of diverse function. However, the strict subcellular segregation of other MAPs in brain may be determined by other factors.

Key words: tubulin/microtubules/cerebellum/neurons/cytoskeleton

Introduction

Although microtubules are involved in a diverse variety of cellular functions (e.g. mitosis, cell motility and intracellular transport) the molecular features and/or interactions which distinguish one functionally distinct kind of microtubule from another are completely unknown. In vertebrates, the major components of microtubules are present in multiple forms due to the expression of multigene families for both α- and β-tubulin (Cowan, 1984; Cleveland and Sullivan, 1985) and as a result of variations in the extent of post-translational modifications (Gundersen *et al.*, 1984; Piperno and Fuller, 1985; Cambray-Deakin and Burgoyne, 1987a,b). In addition, a range of microtubule types could be generated by differential association of microtubule-associated proteins (MAPs). The importance of tubulin isotypes derived from multiple genes in the generation of microtubule diversity is unclear. It is known that in some circumstances microtubules can form which are co-polymers of several β-tubulin isotypes (Bond *et al.*, 1986; Lewis *et al.*, 1987; Lopata and Cleveland, 1987). Six mammalian β-tubulin genes have been identified which represent most if not all of the expressed genes for β-tubulin (Bond *et al.*, 1984; Lewis *et al.*, 1985a,b; Sullivan and Cleveland, 1986; Wang *et al.*, 1986; Cowan *et al.*, 1988). In the mouse one β-tubulin isotype (Mβ1) is expressed only in haematopoietic tissues (Wang *et al.*, 1986) but the remaining five isotypes are expressed in brain with Mβ4 being restricted to this tissue (Cowan *et al.*, 1988). In order to gain some insight into the importance of β-tubulin diversity it is of interest to determine whether β-tubulin isotypes are expressed indiscriminately in all cell types in brain or whether they show any differential cellular or subcellular localization.

The cerebellum has a well-documented laminated organization (Palay and Chan-Palay, 1974) that has allowed the distribution of several microtubule proteins, including post-translationally modified forms of α-tubulin (Cumming *et al.*, 1984; Cambray-Deakin and Burgoyne, 1987a), to be located by immunocytochemistry. A number of MAPs have been localized in cerebellum and shown to have restricted and characteristic distributions (Burgoyne and Cambray-Deaking, 1988). An examination of the localization of β-tubulin isotypes in cerebellum offers the possibility of examining the relationship of their distribution to that of MAPs. This is of interest since, with the exception of Mβ1, the majority of the sequence differences between the β-tubulin isotypes lie within a variable 15-amino-acid carboxy-terminal region (Bond *et al.*, 1984; Lewis *et al.*, 1985b; Sullivan and Cleveland, 1986; Wang *et al.*, 1986). The carboxy-terminal region of β-tubulin has been shown to be important in MAP binding to microtubules (Serrano *et al.*, 1984a; Littauer *et al.*, 1986) and it has been suggested that different β-tubulin isotypes may bind distinct MAPs (Lewis *et al.*, 1985b, 1987). The results presented are consistent with the idea that the variable carboxy-terminal region of β-tubulin contributes to the control of recruitment of particular MAPs to specific microtubules.

Results

Isolation of a cDNA clone encoding Mβ6 and generation of an Mβ6-specific antiserum

We previously described cloned cDNAs encoding five distinct mouse β-tubulin isotypes (Mβ1, Mβ2, Mβ3, Mβ4 and Mβ5; Lewis *et al.*, 1985a; Wang *et al.*, 1986) and their regulated patterns of developmental expression at the level of mRNA. Four of these isotypes—Mβ2, Mβ3, Mβ4 and Mβ5—are expressed in brain. Recently, however, a human

β-tubulin isotype (designated hβ4) was described by Sullivan and Cleveland (1986) which, by analogy with a highly homologous chicken β-tubulin isotype (cβ4), was presumed to be neuron specific. Based on the strict interspecies conservation of mammalian tubulin isotypes (Villasante *et al.*, 1986; Wang *et al.*, 1986) we reasoned that a corresponding neuron-specific isotype should exist in the mouse. Several cDNA clones encoding this isotype, which we have termed Mβ6, were isolated. Sequencing shows that there are two amino acid differences between this isotype and its human counterpart, hβ4: amino acid 442 is glutamic acid in hβ4 and aspartic acid in Mβ6, while amino acid 446 is serine in hβ4 and arginine in Mβ6. While Mβ6 differs in only 0.5% of its amino acid sequence from its human counterpart, it differs from the other four mouse tubulins expressed in brain by 9−10%. Most of these differences occur in the extreme carboxy-terminal amino acids (Figure 1). The full sequence of the Mβ6 cDNA is available upon request.

We recently described the generation of β-tubulin isotype-specific antisera that discriminate among the isotypes encoded by Mβ2, Mβ3 plus Mβ4 and Mβ5 (Lewis *et al.*, 1987), and showed that, at least in cells in culture, there is free intermingling of these naturally occurring isotypes in functionally distinct microtubules. Similar data have since been obtained by Lopata and Cleveland (1987). To extend our collection of isotype-specific sera to include Mβ6, we assembled a chimaeric β-tubulin sequence containing the carboxy-terminal 105 amino acids of this isotype, and expressed this chimaeric construct as a lac Z fusion protein in host *Escherichia coli* cells. This fusion protein was used as an immunogen in rabbits which had been rendered tolerant to fusion proteins corresponding to Mβ2, Mβ3, Mβ4 and Mβ5; this procedure effectively eliminates any response to epitopes that are shared among the different β-tubulin isotypes (Lewis *et al.*, 1987).

β2	ADEQGEFEEEEGEDEA
β3	AEEEGEFEEEAEEEVA
β4	AEEGEFEEEAEEEVA
β5	AEEEEDFGEEAEEEA
β6	AEEEGEMYEDDDEESERQGPK

Fig. 1. Comparison of the carboxy-terminal sequences of five mouse β-tubulin isotypes that are expressed in brain. Data from Lewis *et al.* (1985a; Mβ2, Mβ4, Mβ5), Wang *et al.* (1986; Mβ3) and this paper (Mβ6).

The specificity of the antibody raised against Mβ6 is shown in Figure 2A,B in which the affinity-purified antibody was tested by Western blotting against whole bacterial extracts from host cells expressing cloned fusion proteins encoding Mβ1, Mβ2, Mβ3, Mβ4, Mβ5 and Mβ6. The data show that the Mβ6 antibody detects only the fusion protein corresponding to this isotype (Figure 2B, track 7); no reaction is evident with fusion proteins corresponding to any other isotype. Marker brain β-tubulin is also detected by the Mβ6 antibody (Figure 2B, track 1), as a consequence of the expression of this isotype in brain tissue (see below). However, the reaction with brain β-tubulin is weak due to the low level of expression of Mβ6 in brain relative to other isotypes. The Mβ6 antibody was used to probe Western blots of whole extracts of adult mouse ovary, testis, brain and spleen (Figure 2C). In this experiment, the Mβ6 antibody uniquely detects β-tubulin in brain (strongly) and testis (weakly), but not in spleen or ovary.

Expression of Mβ6 in developing and adult mouse tissues

To assess the overall pattern of expression of Mβ6 in adult and developing mouse tissues, a restriction fragment containing only 3′-untranslated region sequences corresponding to Mβ6 was used to probe RNA prepared from adult brain, heart, kidney, liver, lung, muscle, spleen, stomach, testis and thymus (Figure 3A). The sequences contained in this 3′-untranslated region fragment bear no detectable homology to the corresponding regions of any other mouse β-tubulins. The data show expression of Mβ6 in brain and (weakly) in testis, but not in any other tissues. In an analogous experiment using mouse brain RNA from animals aged 3, 6, 10, 15, 22 and 32 days (Figure 3B) there is a marked decline in the expression of Mβ6 with increasing

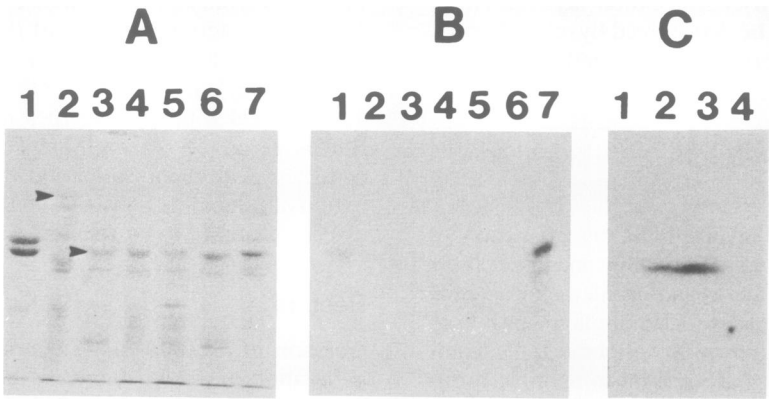

Fig. 2. Specificity of an isotype-specific antibody to Mβ6. (**A**) Coomassie blue stain of an 8.5% SDS−polyacrylamide gel loaded with marker brain tubulin (**track 1**) and whole extract of bacterial cells expressing fusion proteins corresponding to Mβ1 (**track 2**), Mβ2 (**track 3**), Mβ3 (**track 4**), Mβ4 (**track 5**), Mβ5 (**track 6**) and Mβ6 (**track 7**). Fusion proteins are arrowed. (**B**) Immunoblot of a gel identical to that shown in (A) reacted with an affinity-purified antibody raised against the fusion protein encoding Mβ6. (**C**) Immunoblot of an 8.5% SDS−polyacrylamide gel loaded with whole extracts of mouse ovary (**track 1**), testis (**track 2**), brain (**track 3**) and spleen (**track 4**). The staining intensities in (B) and (C) cannot be directly compared since these derive from separate experiments.

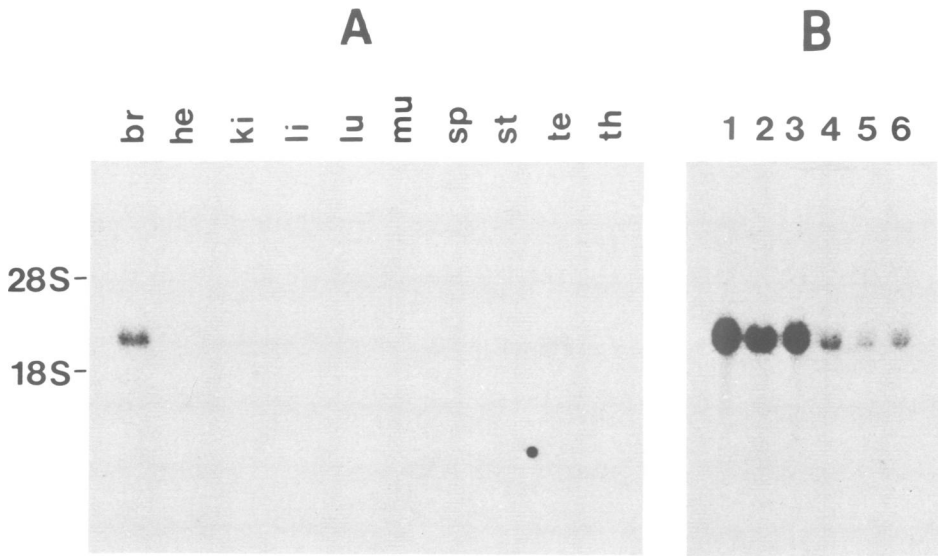

Fig. 3. Expression of Mβ6 in developing and adult mouse tissues. (**A**) Total RNA was prepared from brain (br), heart (he), kidney (ki), liver (li), lung (lu), muscle (mu), spleen (sp), stomach (st), testis (te) and thymus (th) of adult mice. Aliquots (20 μg) of each RNA sample were resolved on denaturing 1% agarose gels and the gel content transferred to nitrocellulose. The blot was probed with a *BglI*−*Eco*RI restriction fragment derived from the 3′-untranslated region of Mβ6 labelled with ^{32}P by nick translation. Following hybridization, the blot was washed to a final stringency of 2 × SSC, 60°C. (**B**) Total RNA was prepared from the brains of young mice aged 3, 6, 10, 15, 22 and 32 days. Aliquots (10 μg) of these preparations were analysed in an RNA blot transfer experiment exactly as described above.

age. No expression of Mβ6 was detected in any of eight other developing somatic tissues, but a very low and essentially constant level of expression was apparent in immature testis at days 10, 15, 22 and 32 (data not shown).

Localization of β-tubulin isotypes in rat cerebellum

The specificity of the antiserum against Mβ6 is demonstrated above and the specificity of the antisera against Mβ1, Mβ2, Mβ3/4 and Mβ5 has been described previously (Lewis *et al.*, 1987). Apart from the reactivity of anti-Mβ3/4 with both Mβ3 and Mβ4 there was no reaction of the affinity-purified antisera with more than a single isotype. The epitopes recognized by the antisera lie in the carboxy-terminal regions of the β-tubulin isotypes (N.J.Cowan, unpublished results). We examined the localization of β-tubulin isotypes in adult rat cerebellum using affinity-purified antisera specific for Mβ1, Mβ2, Mβ3/4, Mβ5 and Mβ6 in comparison with a monoclonal anti-β-tubulin antibody (Tu 2.5) that gives a general staining pattern indistinguishable from polyclonal antisera recognizing both α- and β-tubulin. Mβ1 is not expressed in brain (Wang *et al.*, 1986) and therefore the antiserum specific for this isotype acted as a control in the immunocytochemistry; no staining with this antiserum was detected in brain sections (Figure 4).

Each of the antisera specific for Mβ2, Mβ3/4, Mβ5 and Mβ6 gave distinctive staining patterns in cerebellar sections that differed from each other and from the general staining pattern with Tu 2.5 (Figure 5). Anti-Mβ2 stained Bergmann glial processes in the molecular layer (Figure 5b). Neuronal elements in the molecular and granular layers were unstained. In white matter anti-Mβ2 stained glial cell bodies and axons. Neurons of the deep cerebellar nuclei were the only neuronal cell type stained by this antiserum (Figure 6b). Anti-Mβ3/4 stained granule cell bodies and their parallel fibres and Bergmann glial processes (Figure 5c). In the white

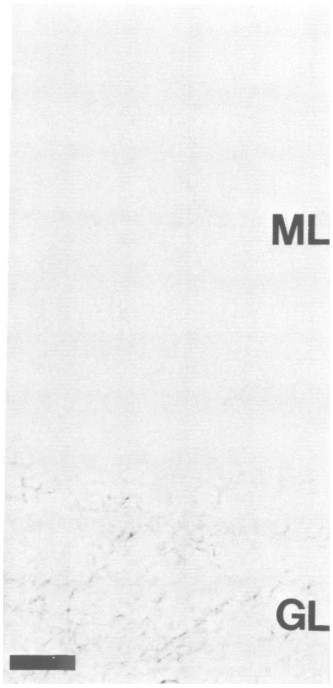

Fig. 4. Control vibratome section of adult rat cortex stained with anti-Mβ1. Mβ1 is not expressed in brain and no staining was detected with this antibody in molecular layer (ML) or granular layer (GL). The section was processed in parallel with those in Figure 5. Scale bar = 25 μm.

matter no staining was detectable in axons but astrocytes were strongly stained (Figure 6c). Neurons of the deep cerebellar nuclei were unstained by anti-Mβ3/4 (Figure 6c). Anti-Mβ5 gave low levels of staining in granule cell bodies and parallel fibres, but the most prominent feature of this

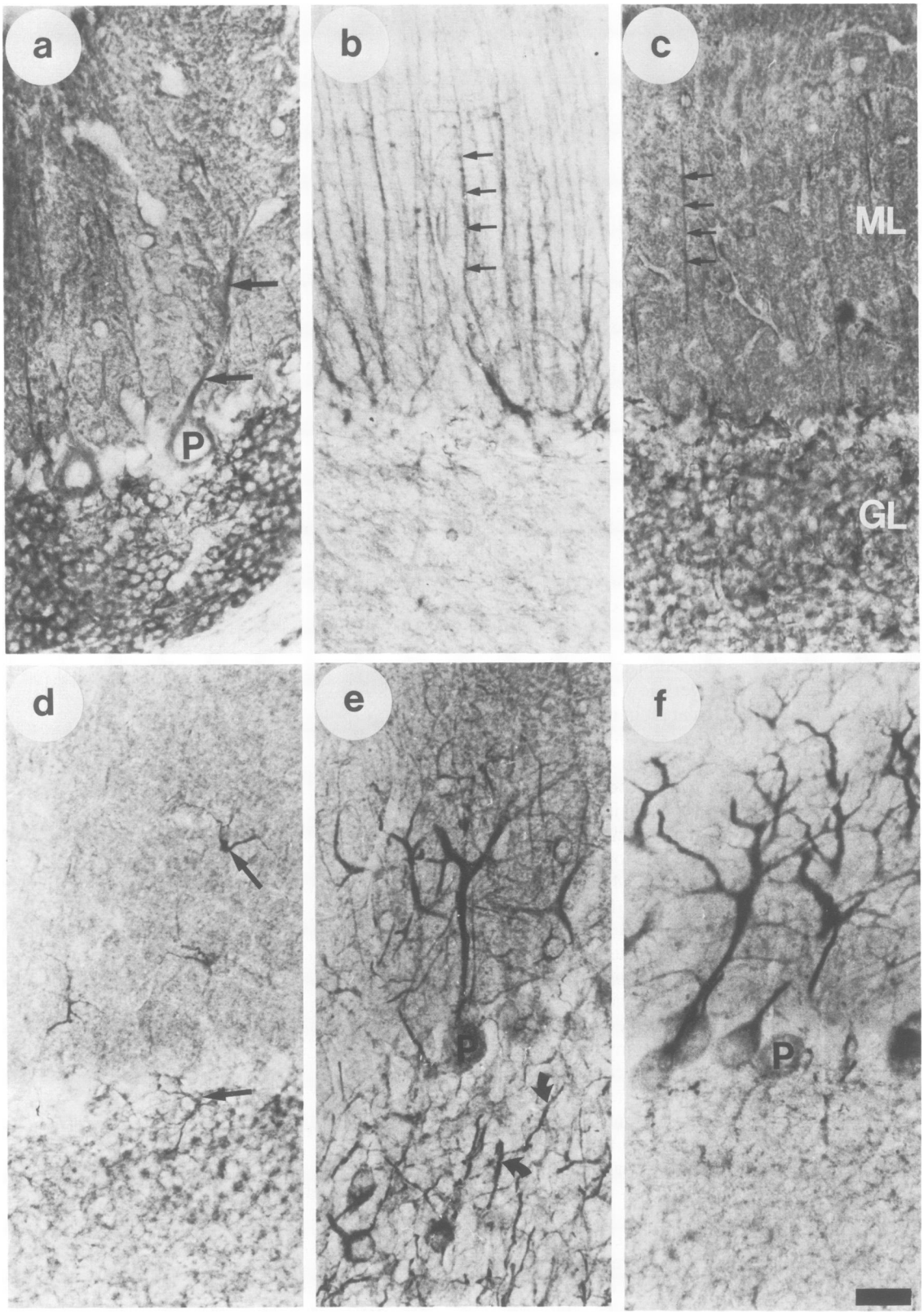

Fig. 5. Immunoperoxidase staining of vibratome sections of adult rat cerebellar cortex. Sections were stained with the general anti-β-tubulin monoclonal Tu 2.5 (**a**), anti-Mβ2 (**b**), anti-Mβ3/4 (**c**), anti-Mβ5 (**d**), anti-Mβ6 (**e**) and anti-MAP1A (**f**). Tu 2.5 gives a general staining pattern including staining of Purkinje cell bodies (P) and dendrites (arrows) and punctate staining in the molecular layer of parallel fibres. Anti-Mβ2 staining is almost entirely restricted to Bergmann glial fibres (arrows in **b**). Anti-Mβ3/4 stains parallel fibres in the molecular layer (ML) and granule cell bodies in the granular layer (GL) and Bergmann glial fibres (arrows in **c**). Anti-Mβ5 stains parallel fibres and granule cell bodies and gives intense staining of a class of presumed protoplasmic astrocytes (arrows in **d**). Anti-Mβ6 stains Purkinje cells and their dendrites and in the granule layer gives strong staining of axons (curved arrows in **e**). The staining with anti-MAP1A is most intense in Purkinje cells and their dendrites (**f**). Scale bar = 25 μm.

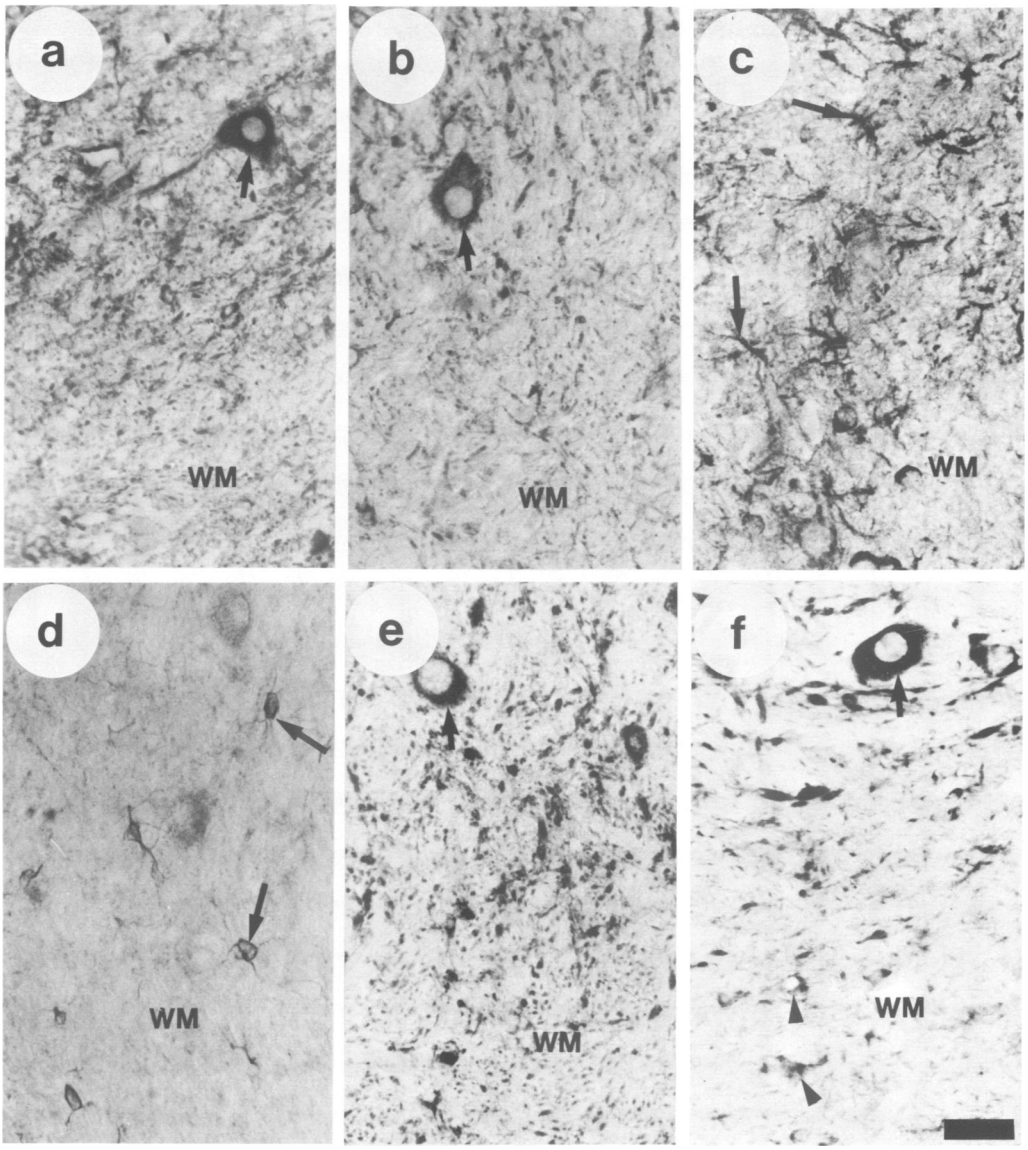

Fig. 6. Immunoperoxidase staining of vibratome sections of adult rat cerebellar white matter and deep cerebellar nuclei. Sections were stained with Tu 2.5 (**a**), anti-Mβ2 (**b**), anti-Mβ3/4 (**c**), anti-Mβ5 (**d**), anti-Mβ6 (**e**) and anti-MAP1A (**f**). Tu 2.5 and anti-Mβ2 (**a,b**) stain neuronal cell bodies (arrows) and axons in the white matter (WM). Anti-Mβ3/4 primarily stains astrocytes within the deep cerebellar nucleus (arrows in **c**). Anti-Mβ5 stains only a class of cells with a stellate morphology (presumed protoplasmic astrocytes) in the deep cerebellar nucleus and white matter (**d**). Anti-Mβ6 stains neuronal cell bodies (arrow in **e**) and gives intense staining of axons in the white matter. Anti-MAP1A stains neuronal cell bodies (arrows in **f**), glial cell bodies (arrowheads) and some axons in the white matter. Scale bar = 25 μm.

antiserum was the staining of cells with a stellate morphology sparsely distributed in the molecular layer, Purkinje cell layer, granular layer (Figure 5d) and white matter. Near the deep cerebellar nuclei, cells of this morphology were the only structures stained; white matter axons were unstained (Figure 6d). The cell types strongly stained by anti-Mβ5 were essentially identical in morphology, number and distribution to a recently described class of $GFAP^-$ protoplasmic astrocytes (Levine and Levine, 1987). The protoplasmic astrocytes were also detected in the cerebral cortex and cells with this morphology were stained by anti-Mβ5 in sections of cerebral cortex (not shown).

None of the preceding β-tubulin isotype-specific antisera stained Purkinje cells or their dendrites. However, Purkinje cells were stained by anti-Mβ6 (Figure 5e), indicating that this class of cells expresses only a single known β-tubulin isotype. Mβ6 was also present in parallel fibres at low levels but was undetectable in granule cell bodies. In the granular layer, Golgi cells and their dendrites were stained, as well as synaptic glomeruli. Anti-Mβ6 also stained numerous processes in the granular layer. These processes had a morphology and distribution essentially identical to that of axons arising from the white matter stained with an antibody against the 200-kd neurofilament protein (data not shown). Anti-Mβ6 stained the neuronal cell bodies in the deep cerebellar nuclei and axons of the white matter (Figure 6e). No evidence of staining of any glial cells was seen with anti-Mβ6. Therefore, Mβ6 appears to be neuronal specific. The distribution of Mβ6 resembled that of MAP1A in being expressed in Purkinje cells and white matter axons (Bloom *et al.*, 1984; Huber and Matus, 1984; Cambray-Deakin *et al.*, 1987). For that reason we stained sections from the same

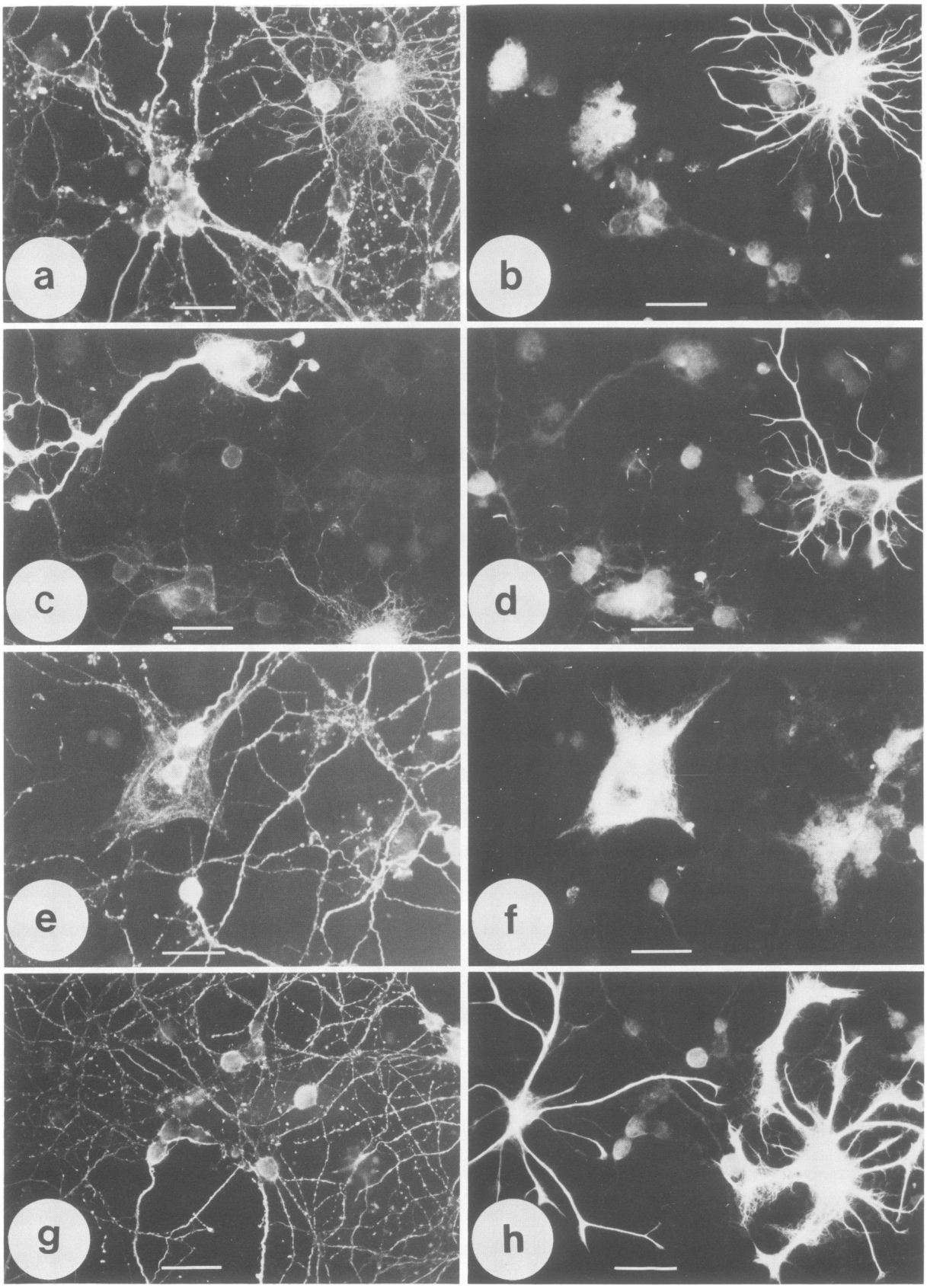

Fig. 7. Immunofluorescence staining of rat cerebellar cultures with β-tubulin isotype-specific antisera and anti-GFAP. Cerebellar cultures were double-labelled with isotype-specific antisera (**a**,**c**,**e**,**g**) and anti-GFAP (**b**,**d**,**f**,**h**). Anti-Mβ2 (**a**) stained neuronal cell bodies and processes and astrocytes (compare **a** and **b**). Anti-Mβ3/4 (**c**) stained neurons faintly and gave bright staining of a class of cells unstained by anti-GPAF (**d**). Anti-Mβ5 (**e**) stained neuronal cell bodies and processes and astrocytes. Anti-Mβ6 (**g**) staining was restricted to neuronal cell bodies and processes; astrocytes were unstained (compare **g** and **h**). Scale bar = 20 μm.

tissue block with a monoclonal antibody specific for MAP1A. Anti-MAP1A stained Purkinje cell bodies and dendrites strongly. In contrast to Mβ6, anti-MAP1A did not stain parallel fibres and axons in the granular layer were unstained (Figure 5f). In addition, glial cell bodies in the white matter which were unstained by anti-Mβ6 were stained by anti-MAP1A (Figure 6f).

In order to examine whether changes in β-tubulin isotype expression occur during cerebellar development we examined the localization of β-tubulins in sections from immature cerebellum. The major findings were as follows (data not shown). First, Mβ2 was transiently expressed in immature parallel fibres. Second, despite its more general distribution in adult cerebellum, anti-Mβ3/4 gave an entirely glial-specific staining pattern in P15 cerebellum with staining restricted to Bergmann glial processes and astrocytes of the granular layer and glial cells of the white matter. Third, Mβ6 was the only isotype present in Purkinje cells and as in the adult cerebellum this isotype was restricted to neurons.

Localization of β-tubulin isotypes in cerebellar cultures

In order to examine the cell-type specificity of β-tubulin isotype expression in immature cerebellar cells in more detail, we examined the distribution of β-tubulin isotypes in primary cultures of cells dissociated from the early postnatal cerebellum. These cultures contain mainly granule cell neurons (Currie, 1980) which are morphologically distinct from non-neuronal cells in the cultures. The cultures were double-labelled with antisera specific for each of the β-tubulin isotypes and with either anti-glial fibrillary acidic protein (anti-GFAP) as a marker for astrocytes or anti-galactocerebroside as a marker for oligodendrocytes. Mβ2 was found to be expressed by all cell types in culture, which is consistent with a general distribution in sections of immature cerebellum (Figure 7a,b). Anti-Mβ3/4 gave strong staining of a sub-population of non-neuronal cells which were not stained by anti-GFAP (Figure 7c,d) but were stained by anti-galactocerebroside, indicating that they were oligodendrocytes (not shown). In addition, anti-Mβ3/4 stained astrocytes of an epitheloid but only few of stellate morphology and weakly stained the cell bodies and processes of granule cells. The Mβ5 isotype was present in cell bodies and processes of granule cells and also anti-GFAP stained cells of epitheloid and stellate morphology (Figure 7e,f). Mβ6 appeared to be entirely neuronal specific; anti-Mβ6 stained the cell bodies and processes of all granule cells but no other cell type was stained (Figure 7g,h).

Discussion

We have previously characterized four β-tubulin isotypes—Mβ2, Mβ3, Mβ4 and Mβ5—that are expressed in mouse brain (Lewis *et al.*, 1985a; Wang *et al.*, 1986). Here we describe the isolation of a fifth isotype, Mβ6, that is abundantly expressed in developing mouse brain, declining to a lower level in the adult; the same isotype is expressed (though to a much lesser extent) in testis. Comparison of α- and β-tubulin isotypes from several mammalian species shows that the amino acid sequences are in most cases identical or almost identical (Lewis *et al.*, 1985a; Villasante *et al.*, 1986; Wang *et al.*, 1986), the only significant exception being the haematopoietic isotype, Mβ1, which is in any case not expressed in brain. Because of this identity, the isotype-specific sera we raised against mouse fusion proteins (Lewis *et al.*, 1987 and this work) can be used to investigate patterns of isotype-specific β-tubulin expression in brain tissue in any mammalian species.

Determination of the functional significance of the expression of multiple tubulin genes in a particular tissue requires information regarding the distribution of the various gene products. The availability of antisera that are able to discriminate among the six known mammalian β-tubulin isotypes has allowed us to determine the pattern of expression of these isotypes in adult rat cerebellum. Because the haematopoietic isotype, Mβ1, is not expressed in brain (Wang *et al.*, 1986), the antiserum specific for this protein acted as a control in the present series of experiments. One antiserum was unable to discriminate between Mβ3 and Mβ4, both of which are expressed in brain (Lewis *et al.*, 1985a; Wang *et al.*, 1986; Cowan *et al.*, 1988), and therefore the staining pattern of this antiserum represented the localization of two separate isotypes. Since the mRNA encoding Mβ4 only appears during late stages of brain development (Lewis *et al.*, 1985a) it is conceivable that the glial-specific staining pattern seen with anti-Mβ3/4 in immature cerebellum is an indication of Mβ3 expression. A definitive answer to the question of Mβ3 and Mβ4 localization will require antisera that are able to discriminate between the two isotypes. We will refer in the rest of the discussion to localization of Mβ3/4 without any prejudice as to which isotype is present at any particular site.

The results presented here show that the β-tubulin isotypes have specific cellular distributions in rat cerebellum and that the distribution of the isotypes changes during cerebellar development. These results suggest that the multiplicity of β-tubulin genes expressed in brain may be of some significance for microtubule function—otherwise there would be no requirement for each particular cell type to have a distinctive complement of β-tubulin isotypes. It seems possible that the presence of particular combinations of isotypes (such as Mβ2 and Mβ3/4 in Bergmann glia) or single isotypes (such as the presence of only Mβ6 in Purkinje cell bodies and dendrites) confers different properties on microtubules in those cells.

With the exception of Mβ1, most of the sequence variation between the β-tubulin isotypes lies within a 15-amino-acid carboxy-terminal region (Figure 1; Bond *et al.*, 1984; Lewis *et al.*, 1985a,b; Sullivan and Cleveland, 1986; Wang *et al.*, 1986). The carboxy terminus of the β-tubulin polypeptide influences microtubule assembly (Serrano *et al.*, 1984b; Sackett *et al.*, 1985) and the binding of microtubule-associated proteins (Serrano *et al.*, 1984a; Littauer *et al.*, 1986). It has previously been suggested that the importance of the expression of multiple β-tubulin genes is that they could confer the ability to assemble sub-classes of microtubules which have different MAPs (Lewis *et al.*, 1985b; Wang *et al.*, 1986). Studies on the localization of several MAPs in rat cerebellum (Bernhardt and Matus, 1984; Bloom *et al.*, 1984, 1985; Burgoyne and Cumming, 1984; Huber and Matus, 1984; Bernhardt *et al.*, 1985; Binder *et al.*, 1985; Parysek *et al.*, 1985; Riederer *et al.*, 1986; Cambray-Deakin *et al.*, 1987) have shown that the MAPs are not present on all classes of microtubules but have restricted cellular and subcellular distributions. We have compared the localization of the β-tubulin isotypes with that of the MAPs previously described. The Mβ2 isotype has a distribution indistinguishable from that reported for MAP3 (Bernhardt *et al.*, 1985).

The Mβ2 isotype is present in Bergmann glial cells, astrocytes and white matter axons in adult cerebellum and is expressed transiently in the parallel fibres in immature cerebellum; this is the pattern of expression described for MAP3 (Bernhardt *et al.*, 1985). In addition, Mβ2 is also present at high levels in astrocytes in the cerebral cortex (our unpublished observations) as described for MAP3 (Riederer and Matus, 1985). The only other similarity between β-tubulin isotype and MAP localization was seen for the Mβ6 isotype. Like MAP1A (Bloom *et al.*, 1984; Huber and Matus, 1984; Cambray-Deakin *et al.*, 1987), Mβ6 was present in Purkinje cells and their dendrites and was also found in white matter axons. However, Mβ6 distribution differed from that of MAP1A since Mβ6, but not MAP1A, was present at high levels in axons in the granular layer. In addition, Mβ6 was neuronal specific in sections and cerebellar cultures but MAP1A was present in glial cell bodies in the white matter and in non-neuronal cells in cerebellar culture (Cambray-Deakin *et al.*, 1987). It seems likely that the expression of multiple β-tubulin isotypes in brain may be partially responsible for the recruitment of particular MAPs to microtubules. However, it is clear that additional factors must be involved in the generation of the differential distribution of MAPs between axonal and dendritic microtubules of the same cells. In the case of the restriction of MAP2 to dendritic (Bernhardt and Matus, 1984; Burgoyne and Cumming, 1984) and tau to axonal (Binder *et al.*, 1985) microtubules, the mechanism may involve post-translational modifications of α-tubulins since both acetylated and detyrosinated α-tubulins are enriched in axons (Cumming *et al.*, 1984; Cambray-Deakin and Burgoyne, 1987a).

Previous work suggests that in any particular cell expressing more than one β-tubulin isotype these isotypes are present in all microtubules (Lewis *et al.*, 1987). The present results indicate that the β-tubulin isotypes have cell-specific patterns of expression in cerebellum. In general there was little indication that the β-tubulin isotypes were restricted to microtubules of any one subcellular compartment of a particular cell type. The only exception was the presence of Mβ6 in parallel fibres but not the granule cell bodies in the adult cerebellum; it is not known whether or not Mβ6 is present in granule cell dendrites. From the studies presented here on cerebellar sections we cannot say whether or not any of the cell types examined that contain more than one β-tubulin isotype have these isotypes present in the same or separate classes of microtubules. It should be noted that we cannot entirely rule out the possibility that some aspects of the apparent cell type-specific localization of the β-tubulin isotypes could result from differences in the tertiary structure of the isotypes or masking of the epitopes by associated proteins in particular cell types.

In conclusion, this study shows that β-tubulin isotypes are expressed in unique cell-type specific patterns in mammalian brain. In two cases these patterns are similar or identical to the patterns of expression of specific MAPs. This co-ordinated expression of MAPs and β-tubulin isotypes is consistent with the notion that MAP binding may be isotype specific. However, as in cultured cells, we found no evidence for the subcellular sorting of β-tubulin isotypes in neurons or glia, whereas, for example, tau and MAP2 are restricted to axons and dendrites respectively. Thus, the distribution of MAPs is not dictated solely by the distribution of β-tubulin isotypes.

Materials and methods

Isolation and sequence of a cDNA clone encoding Mβ6

Duplicate replica filters of a mouse brain cDNA library cloned in λgt11 (Lewis *et al.*, 1985a) were screened using either a cDNA insert encoding chicken β-tubulin (clone pT2, Cleveland *et al.*, 1980) or a mixed probe consisting of 3′-untranslated sequences corresponding to Mβ2, Mβ3, Mβ4 and Mβ5. Clones giving positive hybridization signals with pT2, but not with any of the 3′-untranslated region probes, were isolated, amplified and their inserts subcloned into M13 vectors for sequence analysis by the didexoy chain termination method (Sanger *et al.*, 1980). Several overlapping clones encoding an isotype distinct from Mβ2, Mβ3, Mβ4 and Mβ5 were sequenced, resulting in the determination of the sequence of Mβ6 from the codon for amino acid 45 to ~300 bp 3′ to the stop codon.

RNA blot transfer experiments

RNA was prepared from adult and developing mouse tissues (Chirgwin *et al.*, 1979). RNA concentrations were determined by absorbance at 260 nm. Aliquots (20 μg) were electrophoresed on 1% agarose gels containing 2.2 M formaldehyde. The gel contents were transferred to nitrocellulose (Southern, 1975) and the blots hybridized with a *Bgl*I–*Eco*RI fragment derived from the 3′-untranslated region of the cDNA encoding Mβ6 ^{32}P-labelled by nick translation (Rigby *et al.*, 1977).

Production of isotype-specific sera

The sera specific for Mβ2, Mβ3/4 and Mβ5 were described previously (Lewis *et al.*, 1987). To generate an antiserum specific for Mβ6, a cloned fusion protein was synthesized in host bacterial cells by cloning a *Bam*HI–*Eco*RI fragment encoding Mβ6 sequences 3′ to amino acid 345 into the 5′-*Bal*31-deleted chicken β-tubulin clone pT2 in pUC8 that encodes amino acids 2–445 of a chicken β-tubulin polypeptide (Lewis *et al.*, 1987). Upon transformation of host *E.coli* cells with this construct, a fusion protein was synthesized consisting of the first six amino acids of *E.coli* β-galactosidase, followed by amino acids 2–344 corresponding to the chicken β-tubulin clone pT2, followed by amino acids 345–450 of Mβ6. This fusion protein was gel purified and used to generate affinity-purified Mβ6 antibody from rabbits following induction of immune tolerance to other mouse β-tubulin isotypes (Lewis *et al.*, 1987). Sera were characterized in Western blot experiments using whole extracts of bacterial cultures or of whole mouse tissues. Extracts, were prepared by direct boiling of cells or tissues in SDS sample buffer, and electrophoresis on 8.5% SDS–polyacrylamide gels (Laemmli, 1970). Gel contents were transferred to nitrocellulose, blocked, incubated with anti-Mβ6 (1:20), washed and reacted with ^{125}I-labelled protein A as previously described (Lewis *et al.*, 1987).

Immunocytochemistry on vibratome sections

Cerebella were fixed by perfusion fixation in anaesthetized rats with 4% formaldehyde, 0.25% glutaraldehyde in PBS or by immersion fixation of small blocks of cerebella in 4% formaldehyde in PBS for 18 h. Essentially identical results were obtained with each fixation protocol. Parasagittal sections (50–100 μm) were cut using a vibratome and sections were incubated with 0.3% H_2O_2 for 30 min followed by incubation in 0.1% Triton X-100, 0.3% BSA in PBS (PBT) for 30 min. Sections were incubated overnight at 4°C with affinity-purified rabbit antisera (at dilutions in the range 1:5–1:100), a mouse monoclonal antibody specific for β-tubulin (Tu 2.5; 1:250) or a mouse monoclonal antibody specific for MAP1A (Bloom *et al.*, 1985; 1:50 000). The sections were then incubated with either anti-rabbit–biotin (1:100) or anti-mouse–biotin (1:100), as appropriate, for 60 min followed by horseradish peroxidase–streptavidin (1:100) for 30 min. All antibodies were diluted in PBT. The reaction was developed with diaminobenzidine/H_2O_2, the sections dehydrated in alcohols, cleared in xylene and mounted. Second antibodies, horseradish peroxidase–streptavidin and anti-MAP1A were obtained from Amersham International, Amersham, UK. The specificity of anti-MAP1A (Bloom *et al.*, 1984) was confirmed by Western blotting.

Cell cultures

Cells were dissociated from cerebella of 6-day-old rats by trypsin treatment (Currie *et al.*, 1979; Dutton *et al.*, 1981), resuspended in culture medium and plated at a cell density of $1.75 \times 10^5/cm^2$ on poly-D-lysine-coated glass coverslips. The cells were maintained for 5 days in a medium consisting of Eagle's minimum essential medium containing 10% fetal calf serum, 100 μg/ml gentamycin, 33 mM glucose, 290 μg/ml glutamate, 25 mM KCl.

Immunofluorescence staining of cell cultures

For double-labelling of astrocytes and β-tubulins, cultures were fixed in 4% formaldehyde in PBS and permeabilized by incubation in PBT for

40 min. Cells were incubated with rabbit anti-β-tubulin antisera (at 1:3–1:25) and a mouse monoclonal specific for glial fibrillary acidic protein (1:50) for 2 h. The cultures were then incubated with anti-mouse Texas Red (1:50) and anti-rabbit–biotin (1:100) for 40 min followed by fluorescein–streptavidin (1:50) for 30 min. All reagents were diluted in PBT. For double-labelling of oligodendrocytes and β-tubulins, live cultures were incubated in 3% BSA in PBS for 40 min followed by mouse monoclonal anti-galactocerebroside (1:50) for 60 min. The cultures were then incubated with anti-mouse Texas Red (1:50) for 40 min and fixed in 4% formaldehyde in PBS. The cultures were subsequently processed for anti-β-tubulin staining as above. After staining the coverslips were washed with acid–alcohol at −10°C and mounted in 0.25% 1,4-diazobicyclo[2,2,2]octane, 0.002% *p*-phenylenediamine in glycerol/PBS (9:1). Immunofluorescence was examined using a 63× oil immersion lens on a Zeiss Universal microscope with appropriate filter for fluorescein and Texas Red.

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