Immunocytochemical Demonstration of α-Tubulin Modification during Axonal Maturation in the Cerebellar Cortex

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ABSTRACT   Previous light microscopic immunocytochemical studies using two monoclonal antibodies that recognise α-tubulin (YOL/34 and YL1/2) but differ in their isotypic specificity have shown that the unmyelinated parallel fiber axons in the cerebellar cortex are labeled with only one of the antibodies (YOL/34). We now show that at 10 d postnatally the parallel fibers are labeled with *both* antibodies, and that during development YL1/2 (but not YOL/34) immunoreactivity disappears progressively from parallel fibers in the lower regions of the molecular layer upwards towards the external germinal layer. By ~28 d postnatally, the differential staining pattern of parallel fibers by the antibodies is established throughout the molecular layer. The time course, light microscopic, and ultrastructural staining distribution corresponds to a progressive change in α-tubulin immunoreactivity as the parallel fibers form synaptic contacts. This modification of α-tubulin (which was not observed in Purkinje cell dendrites or Bergmann glia) may be related to the formation of a basic isotype of α-tubulin within parallel fiber axons at maturation.

Tubulin heterogeneity is most strikingly demonstrated in brain (12). Furthermore, the range of α- and β-tubulin isotypes is expressed by single neurons (15) and tubulin heterogeneity increases during brain development (14). The requirement for multiple isotypes may reflect the variety of different functions of microtubules within each neuron during development and at maturity.

The advent of hybridoma technology has allowed monoclonal antibodies to be produced that have specificity for different regions of the tubulin molecule. We have recently shown, using light and electron microscopic immunocytochemistry (11), that two monoclonal antibodies (YL1/2 and YOL/34) have different specificities for isotypes of α-tubulin, and that these antibodies label different populations of axons in the central nervous system. To examine whether these differences are evident during axonal maturation, we studied the unmyelinated parallel fiber axons of the developing cerebellar cortex, which have a well defined postnatal development (1–3). Our results suggest that immunocytochemical techniques with monoclonal antibodies can be used to visualize a modification of α-tubulin in axons that occurs during development.

MATERIALS AND METHODS

Antibody characterization was carried out as described previously (9, 11), using the SDS gel immunoblotting technique. Cerebellar cytosol was prepared from 10-d-old and adult (60-d) rats by homogenization in 5 mM Tris/2 mM EGTA, pH 8.0, and centrifugation at 40,000 g for 60 min at 4°C, and the protein concentration of the cytosol was determined. Microtubules were prepared from adult rat brain using temperature-dependent assembly/disassembly (6). Samples were solubilized and separated by SDS PAGE on a 6% slab gel. The same protein concentration (100 μg) was used for 10 and 60-d cerebellar cytosol tracks. Immediately following electrophoresis, proteins were transferred from the gel onto nitrocellulose paper (0.45 μm; Millipore Corp., Bedford, MA) by transverse electrophoresis (in Tris/glycine/methanol/water [18]) at 10 V for ~17 h. Confirmation of protein transfer was obtained by staining one track of each age with amidoblack, while the remaining tracks were processed with monoclonal antibodies (ascites fractions) using the immunoperoxidase procedure with diaminobenzidine as substrate. Rat monoclonal antibodies YL1/2 and YOL/34 were raised against yeast tubulin by Kilmartin et al. (16) and have previously been characterized as recognizing brain α-tubulin (8–11). Mouse monoclonal antibody Tu 2.1 was raised as detailed by Gozes and Barnstable (13) against brain microtubules and has been shown to specifically detect isotypes of β-tubulin.

Male Wistar rats of 10, 15, 20, 24 and 28 d postnatally together with adults (60 d and older) were transcardially perfused with 4% paraformaldehyde, 0.25% glutaraldehyde in 50 mM cacodylate buffer, pH 7.2. Following postfixation for a minimum of 16 h at 4°C, 20 μm parasagittal sections of the agar-embedded vermis were cut on a vibratome. After extensive washing in phosphate-buffered saline, sections were incubated for 72 h at 4°C with dilutions of primary monoclonal antibodies and processed for light microscopy using the indirect technique (with peroxidase conjugates; Miles Laboratories, Inc., Elkhart, IN) with 4-chloro-1-naphthol as substrate. Rats of 10 and 15 d were also processed with antibodies YL1/2 and YOL/34 for ultrastructural pre-embedding immunocytochemistry, except that diaminobenzidine was used as substrate (11).

RESULTS

The immunoblotting data shown in Fig. 1 demonstrates that antibodies YL1/2 and YOL/34 selectively label α-tubulin from both 10 d and adult cerebellar cytosol, while antibody
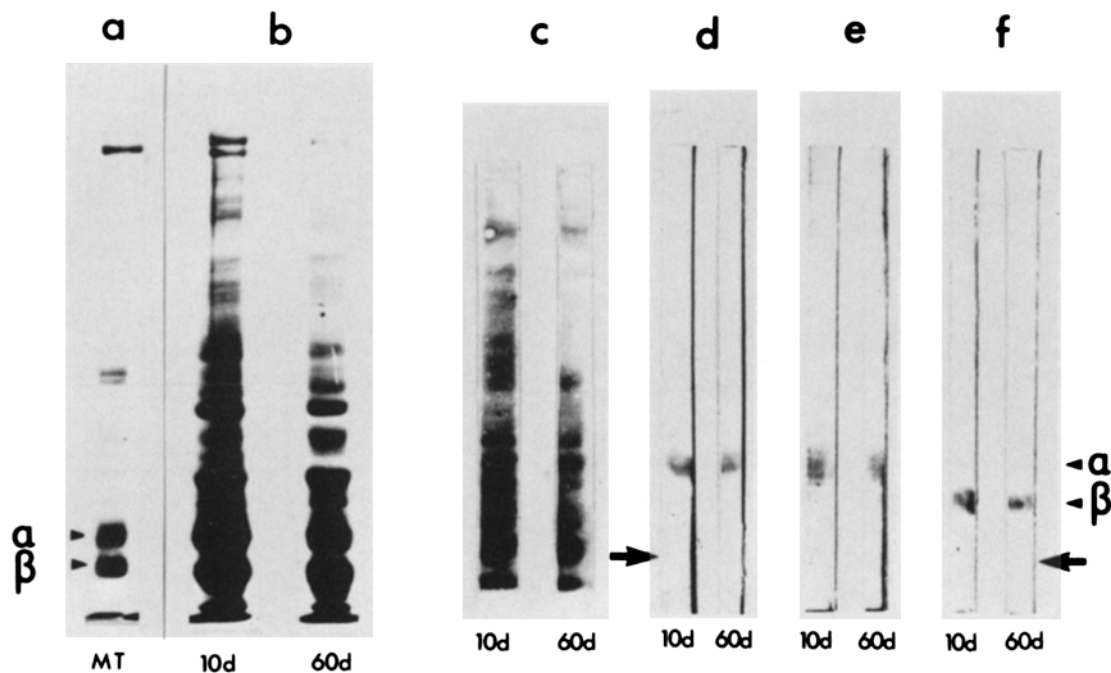
Figure 1 Characterization of monoclonal antitubulin antibodies using 10 d and adult (60 d) cerebellar cytosol. (a and b) Coomassie-Blue-gel tracks of microtubule preparation (MT) and 10 and 60 d cerebellar cytosol. (c–f) Transfer of proteins from SDS PAGE slab gel onto nitrocellulose paper showing confirmation of protein transfer with amidoblack (c) and immunostaining of adjacent tracks with antibodies YL1/2 (d), YOL/34 (e), and Tu 2.1 (f). The immunoblot shows selective staining of single bands corresponding to either α-tubulin (YL1/2, YOL/34) or β-tubulin (Tu 2.1) in both 10-d-old and adult cerebellar cytosol. The position corresponding to the dye front of the gel is indicated by arrows on the blots.

Tu 2.1 labels only the β-tubulin subunit in cerebellar cytosol from rats of both ages. We have previously confirmed that these labeled bands correspond to authentic tubulin subunits by immunoblotting of microtubule preparations (from two cycles of temperature-dependent assembly/disassembly [11]).

Light microscopic immunocytochemistry demonstrated significant differences in the staining of the molecular layer with the antibodies during development (Fig. 2). To eliminate variations between cerebellar folia, we restricted our comparisons to folia in the vermis at the same stages of development.

At 10 d, we observed particulate staining throughout the molecular layer with all of the antibodies, and also found it in the premigratory cell zone of the external germinal layer. The size and distribution of the stained profiles in the molecular layer corresponds to labeling of the parallel fiber axons in transverse section, as described previously using a similar technique (4).

At 15 d, we observed YL1/2 immunoreactivity with a particulate distribution only in the upper region of the molecular layer and also in the premigratory cell zone. Further evidence that these processes represent stained parallel fiber axons comes from coronal sections that show stained longitudinal processes in this region (not shown).

At 20 d, the external germinal layer is only approximately one cell deep, and staining with antibody YL1/2 is confined to a thin layer underneath and between these cells. By 24–28 d, the parallel fiber axons of the molecular layer are completely unstained with antibody YL1/2 reflecting the adult staining pattern (Fig. 2 and reference 11). By contrast, from 10 d onward the parallel fiber axons throughout the molecular layer are labeled with antibodies YOL/34 and Tu 2.1.

Electron microscopic immunocytochemistry at 10 and 15 d confirmed that the particulate labeling in the molecular

layer represented stained parallel fiber axons containing labeled microtubules. Fig. 3 shows parallel fibers labeled with antibody YL1/2 adjacent to the external germinal layer at both ages, in transverse and longitudinal section. We also observed unlabeled axons.

In contrast to the differences described in labeling of the parallel fibers, other stained elements including Purkinje cell dendrites and Bergmann glia showed similar staining with the three antibodies at each developmental stage.

DISCUSSION

Reconciling the immunocytochemical changes described with maturation of the parallel fibers requires comparison with the well defined ultrastructural studies on the developing rat cerebellum (1–3). At 10 d, the molecular layer is densely packed with parallel fiber axons containing abundant microtubules, but the axons lack the varicosities which form synaptic contacts. These immature axons are labeled with both the α-tubulin (YL1/2 and YOL/34) and the β-tubulin (Tu 2.1) antibodies. Between 10 and 21 d, there is a rapid growth in the thickness of the molecular layer, and using electron microscopy parallel fiber axons in the lower regions of the molecular layer have been shown to start to form synaptic contacts. This maturation process, which has been demonstrated using histochemical techniques for Concanavalin A-binding glycoproteins (21) proceeds as a "wave" moving upwards through the molecular layer, as newly formed parallel fibers "stack" on one another. This period correlates with the disappearance of staining with antibody YL1/2. Immature parallel fibers are formed from cytoplasmic growth of the premigratory cells (of the external germinal layer) accounting for staining of these cells and underlying newly formed parallel
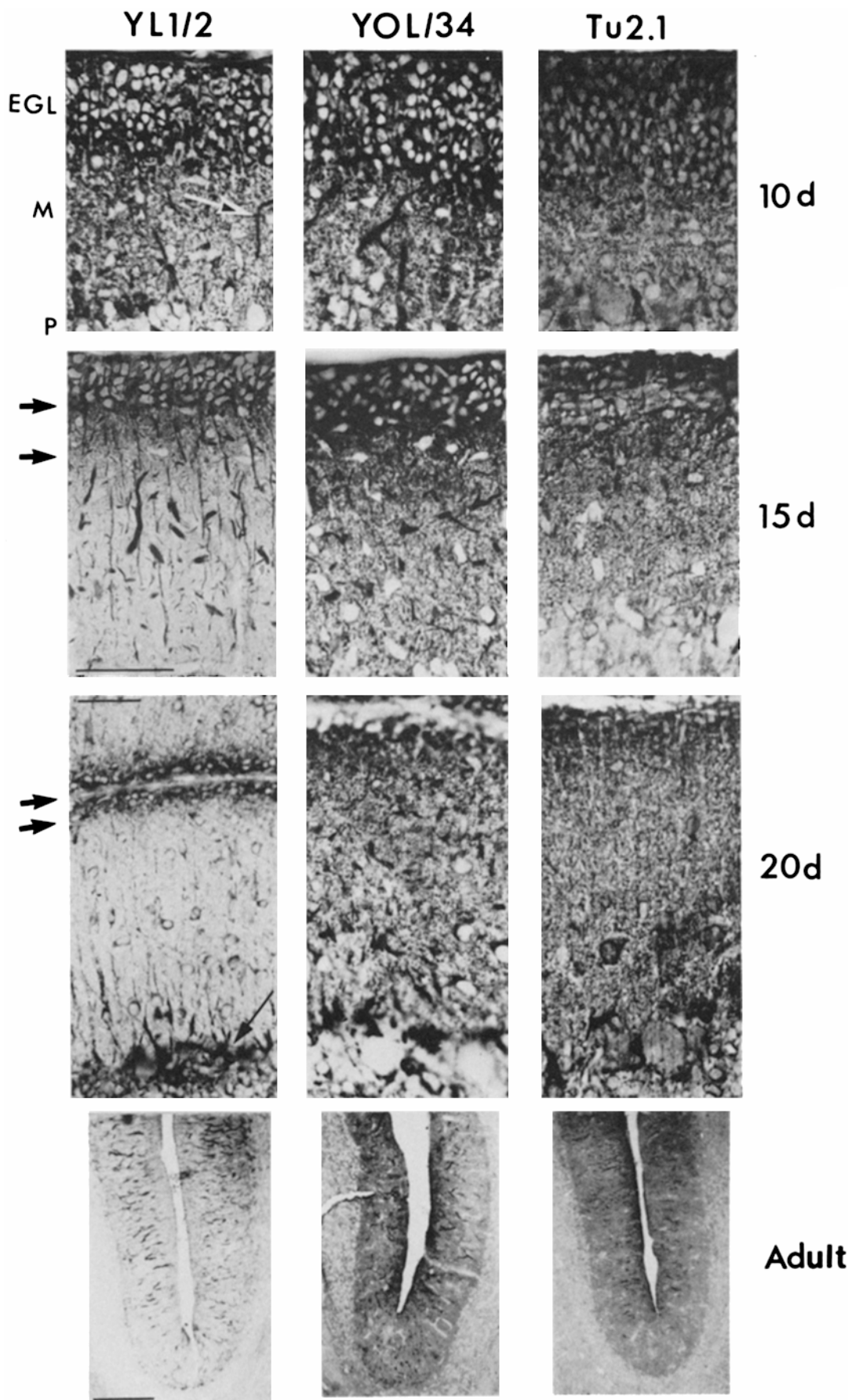
FIGURE 2 Immunoperoxidase localization of α-tubulin (antibodies YL1/2 and YOL/34) and β-tubulin (antibody Tu 2.1) in the cerebellar cortex during postnatal development. EGL, external germinal layer; M, molecular layer; P, Purkinje cell body layer. Particulate labeling of parallel fiber axons in transverse section is seen at 10 d throughout the molecular layer using the three antibodies. At 15 d, labeling with antibody YL1/2 is restricted to a band (between black arrows) in the upper region of the molecular layer, and by 20 d labeling of parallel fiber axons with this antibody is confined to a thin strip close to the pial surface (staining between black arrows). By contrast, parallel fiber axons are stained throughout the molecular layer at all stages of development with antibodies YOL/34 and Tu 2.1. Purkinje cell dendrites (black/white arrows); Bergmann glia (black arrow). Bars: for adults, 200 μm; for YL1/2, 20 d, 50 μm; for remaining micrographs 50 μm. (10 and 15 d) × 330. (20 d) × 210. (Adult) × 50.
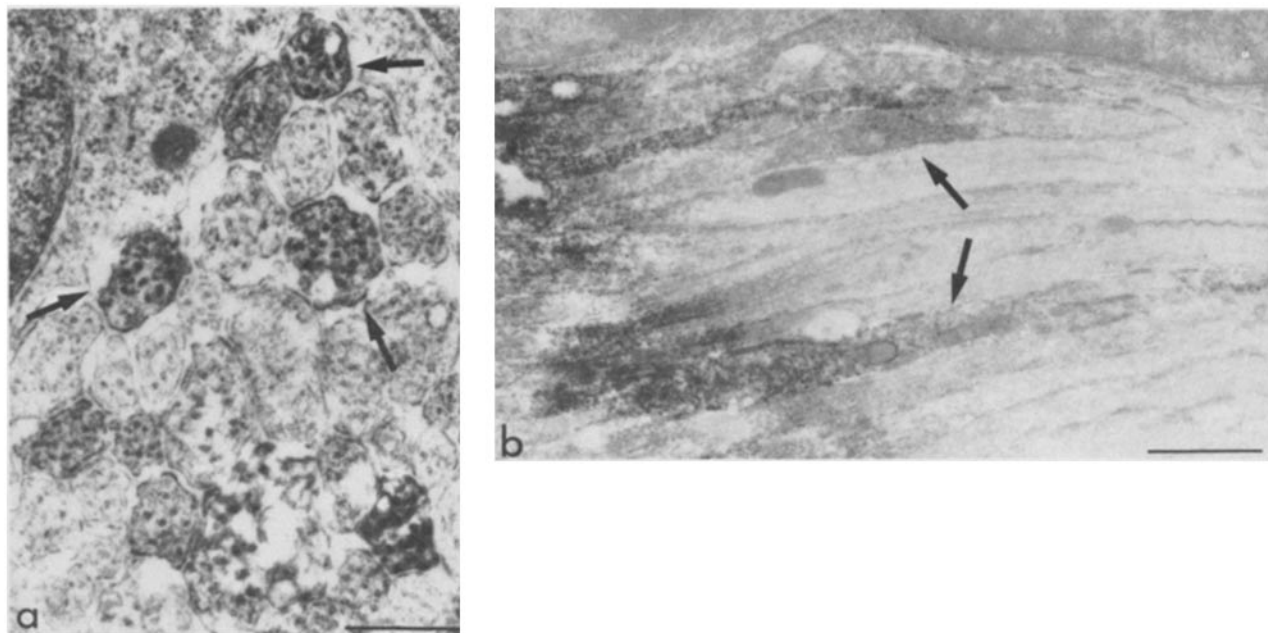
FIGURE 3 Electron microscopic immunoperoxidase localization of α-tubulin (antibody YL1/2) in the molecular layer of the cerebellar cortex (adjacent to the external germinal layer) at 10 d (a) and 15 d (b). Stained parallel fiber axons (arrowed) adjacent to cells in the external germinal layer can be seen in transverse and longitudinal section. Unstained axons are also observed. Bars: (a) 0.5 μm; (b) 1 μm. (a) × 30,000; (b) × 15,000.

fibers with antibody YL1/2 at 15 and 20 d. By 28 d, the parallel fiber axons of the molecular layer are essentially all mature and have formed synaptic contacts with Purkinje cell dendritic spines and other cerebellar neurones (1-3). At this adult stage the parallel fiber axons are completely unlabeled with antibody YL1/2 but labeled with antibodies YOL/34 and Tu 2.1.

We can not be sure at present whether the immunocytochemical changes in α-tubulin during axonal maturation correlate with some aspect of granule cell migration, parallel fiber "stacking" or synaptogenesis. The latter process may be subdivided into early formation of temporary synapses and glial insulation which may be involved in its termination (1-3).

It is interesting that there is no change in the staining of β-tubulin isotypes recognised by antibody Tu 2.1 and that high molecular weight microtubule-associated proteins are apparently not expressed in parallel fiber axons at any stage of development (4, 7). Differences in staining of the antibodies were not observed in Purkinje cell dendrites or Bergmann glia at any stage of development; this would argue against the change in α-tubulin being a common event in all processes at maturation.

While both monoclonal antibodies YL1/2 and YOL/34 have been shown to selectively label brain α-tubulin, differences have been described between the two antibodies. For example, microinjection of antibody YOL/34 into living cells did not detect microtubules, whereas antibody YL1/2 induced a reorganization of microtubules in a variety of cultured cells (19). Recent data has suggested that these differences are due to the antibodies discriminating between tyrosylated and detyrosylated forms of α-tubulin (20).

Using a combination of isoelectric focusing and immunoblotting, we showed recently that antibodies YL1/2 and YOL/34 recognize common and different isotypes of adult brain α-tubulin. Antibody YOL/34 detects more basic isotypes of α-tubulin (11), suggesting that parallel fibers in the adult may

be enriched in these basic isotypes, since these axons are unlabeled with antibody YL1/2. The present results suggest that the tubulin forms recognized by antibody YL1/2 are present in immature but not mature parallel fiber axons. It is of interest that biochemical data shows that there is an increase in a basic isotype of α-tubulin during brain development (14) and that axonal microtubules differ from whole brain microtubules in containing a basic form of α-tubulin associated with the cold-stable population (17).

While the differences in staining with the two monoclonal α-tubulin antibodies during development are compatible with increasing amounts of basic isotypes in mature relative to immature parallel fibers, we can not exclude the possibility of an alternative explanation for our results (e.g., masking of the antigenic determinant of antibody YL1/2 in parallel fibers during development). The modification of α-tubulin could be due to changes in expression of the multiple tubulin genes or posttranslational modification (5, 20) and may regulate the differing functional roles of microtubules in growing and mature axons.

Note Added in Proof: We have recently shown that detyrosylation of α-tubulin by incubation of fixed vibratome sections with pancreatic carboxypeptidase A completely inhibits subsequent immunocytochemical staining by antibody YL1/2 but not by antibody YOL/34. This confirms that antibody YL1/2 only recognizes tyrosylated α-tubulin (20) in cerebellar tissue sections. Therefore the α-tubulin modification described in this paper appears to result from the progressive detyrosylation of α-tubulin in parallel fibres during axonal maturation.

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