

Posttranslational Modifications of α -Tubulin: Acetylated and Detyrosinated Forms in Axons of Rat Cerebellum

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Abstract. The distribution of acetylated α -tubulin in rat cerebellum was examined and compared with that of total α -tubulin and tyrosinated α -tubulin. From immunoperoxidase-stained vibratome sections of rat cerebellum it was found that acetylated α -tubulin, detectable with monoclonal 6-11B-1, was preferentially enriched in axons compared with dendrites. Parallel fiber axons, in particular, were labeled with 6-11B-1

yet unstained by an antibody recognizing tyrosinated α -tubulin, indicating that parallel fibers contain α -tubulin that is acetylated and detyrosinated. Axonal microtubules are known to be highly stable and the distribution of acetylated α -tubulin in other classes of stable microtubules suggests that acetylation and possibly detyrosination may play a role in the maintenance of stable populations of microtubules.

MICROTUBULES are believed to be involved in numerous functions in the adult and developing brain, including cell division, cell migration, intracellular transport along neuronal processes (32), the maintenance and growth of axons (33, 36), and the determination of cellular morphology (26). The heterogeneous functions of microtubules are likely to be subserved by a diversity of microtubule types. This diversity could be generated by assembly of microtubules bearing differing complements of associated proteins (MAPs)¹ or by differences in the forms of α - and β -tubulin subunits within the microtubule polymer. Multiple forms of α - and β -tubulin occur in brain as a result of the expression of multiple genes (11), and a series of posttranslational modifications including phosphorylation of β -tubulin (19), reversible detyrosination/tyrosination of α -tubulin (27, 34), and acetylation of the ϵ -amino group of lysines of α -tubulin (29).

Studies on the localization of MAPs in brain have demonstrated clear differences in the composition of microtubules, both between cell types and between axonal and dendritic processes of the same neurons (6, 30). MAP 2 is largely restricted to neurons, and is present on dendritic but not axonal microtubules (1, 7, 17). Conversely, the tau polypeptides are enriched in axonal microtubules (3). MAP 1 is present in both axons and dendrites but is enriched in dendrites (4, 24). A further protein, MAP 3, is present at high levels in glial cells in adult brain but is expressed transiently in growing axons (2).

Little is known about the distribution of the various isoforms of α - and β -tubulin in brain. We have previously investigated the localization of tyrosinated α -tubulin and found that it is present in dendritic but is absent or depleted from

axonal microtubules in adult brain (8, 13). Tyrosinated α -tubulin is, however, transiently expressed by developing axons (14). A monoclonal antibody specific for acetylated α -tubulin has recently been described by Piperno and Fuller (31). Using this antibody we have investigated the distribution of this posttranslationally modified form of α -tubulin in rat brain. Acetylated α -tubulin is present in both neuronal and nonneuronal cells and appears to be co-distributed, with detyrosinated α -tubulin being preferentially enriched in axons compared with dendrites.

Materials and Methods

Polyacrylamide Gel Electrophoresis and Immunoblotting

Cerebella from adult Wistar rats or pups aged 6, 10, or 18 d were homogenized in 5 mM Tris HCl, 2 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, pH 8.0. Aliquots (100 μ g of protein) were separated by SDS PAGE on 10 or 12.5% slab gels and transferred to nitrocellulose paper by transverse electrophoresis. Protein transfer was confirmed by staining with 0.2% Ponceau S. For immunoblotting, nitrocellulose sheets were washed with PBS, incubated with 3% BSA/0.2% Triton X-100/PBS for 15 min, 6-11B-1 at a 1:10 dilution or YOL/34 at 1:5,000 for 60 min, anti-mouse-biotin or anti-rat-biotin (as appropriate) at 1:600 for 60 min and horseradish peroxidase-streptavidin 1:600 for 30 min. The reaction was developed with diaminobenzidine/ H_2O_2 .

Cell Culture

For granule cell-enriched cultures, cerebellae of 6-d-old rats were dissociated by trypsin treatment (16, 18) plated at a cell density of 1.4×10^3 per mm^2 on poly-L-lysine-coated coverslips and maintained in DME, 30 μ g/ml insulin, 30 nM sodium selenite, 33 mM glucose, 290 μ g/ml glutamine, 25 mM KCl, 25 U/ml penicillin, 25 μ g/ml streptomycin.

Immunofluorescent Staining of Cell Cultures

Cultures were fixed in 4% formaldehyde in PBS and permeabilized by incu-

1. Abbreviations used in this paper: MAP(s), microtubule-associated protein(s); PBT, 0.1% Triton X-100, 0.3% BSA in PBS.

bation in 0.1% Triton X-100, 0.3% BSA in PBS (PBT) for 40 min. Cells were incubated with 6-11B-1 at 1:10. Control coverslips were incubated with nonimmune mouse sera. The cultures were then incubated with anti-mouse Texas red (1:50) for 30 min. All reagents were diluted in PBT. 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 \times oil immersion lens on a Zeiss Universal microscope with the appropriate filters for Texas red fluorescence.

Immunocytochemistry on Vibratome Sections

Cerebella were fixed by immersion in 4% formaldehyde on PBS for 18 h and 50–100 μm parasagittal sections were cut using a vibratome. Sections were incubated in PBT for 30 min followed by 6-11B-1 (1:10), YL1/2 (1:1,000), or YOL/34 (1:1,000) overnight at 4°C . The sections were then incubated in either anti-mouse-biotin (1:100) or anti-rat-biotin (1:100), as appropriate, for 60 min followed by horseradish peroxidase-streptavidin (1:150) 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 in D.P.X. (BDH Chemicals, Ltd., Dagenham, United Kingdom).

Antibodies

The mouse monoclonal 6-11B-1 (culture supernatant) against acetylated α -tubulin (31) was a gift from Dr. G. Piperno (The Rockefeller University, New York) and the rat monoclonals YL1/2 and YOL/34 (25) were gifts from Dr. J. Kilmartin (Cambridge University, Cambridge, England). Rabbit antisera against tyrosinated (anti-Tyr) and detyrosinated (anti-Glu) α -tubulin were a gift from Dr. J. C. Bulinski (University of California at Los Angeles). Second antibodies and horseradish peroxidase-streptavidin were purchased from Amersham International, Amersham, United Kingdom.

Results

In this study we have used three monoclonal antibodies specific for α -tubulin: the mouse monoclonal 6-11B-1, which is specific for acetylated α -tubulin (acetyl-antibody, reference 31); the rat monoclonal YL1/2, which is specific for tyrosinated α -tubulin (tyr-antibody, reference 35); and the rat monoclonal YOL/34 which recognizes all α -tubulin iso-types (general antibody, reference 13).

Immunoblotting with 6-11B-1 (Acetyl Antibody)

The monoclonal 6-11B-1 was raised against sea urchin axonemes and recognizes acetylated α -tubulin in the axonemes of a range of species (31). From immunoblotting of cerebellar homogenates (Fig. 1) it is apparent that the acetyl-antibody recognizes a polypeptide corresponding to rat brain α -tubulin. The staining with acetyl-antibody was consistently weaker than that with the general antibody in parallel experiments (Fig. 1), suggesting that acetylated α -tubulin may be only a small proportion of total brain α -tubulin.

Immunocytochemical Localization of Acetylated α -Tubulin in Adult and Developing Cerebellum

In vibratome sections of adult cerebellum the acetyl-antibody gave intense particulate staining within the molecular layer (Fig. 2 *a*) indicative of staining of granule cell axons (parallel fibers) cut in cross section. Granule cell bodies in the granular layer were also stained. Axons and glial cell

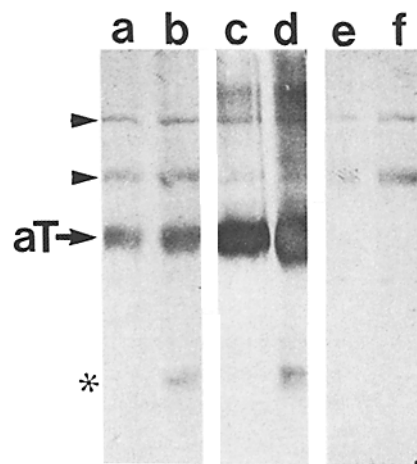


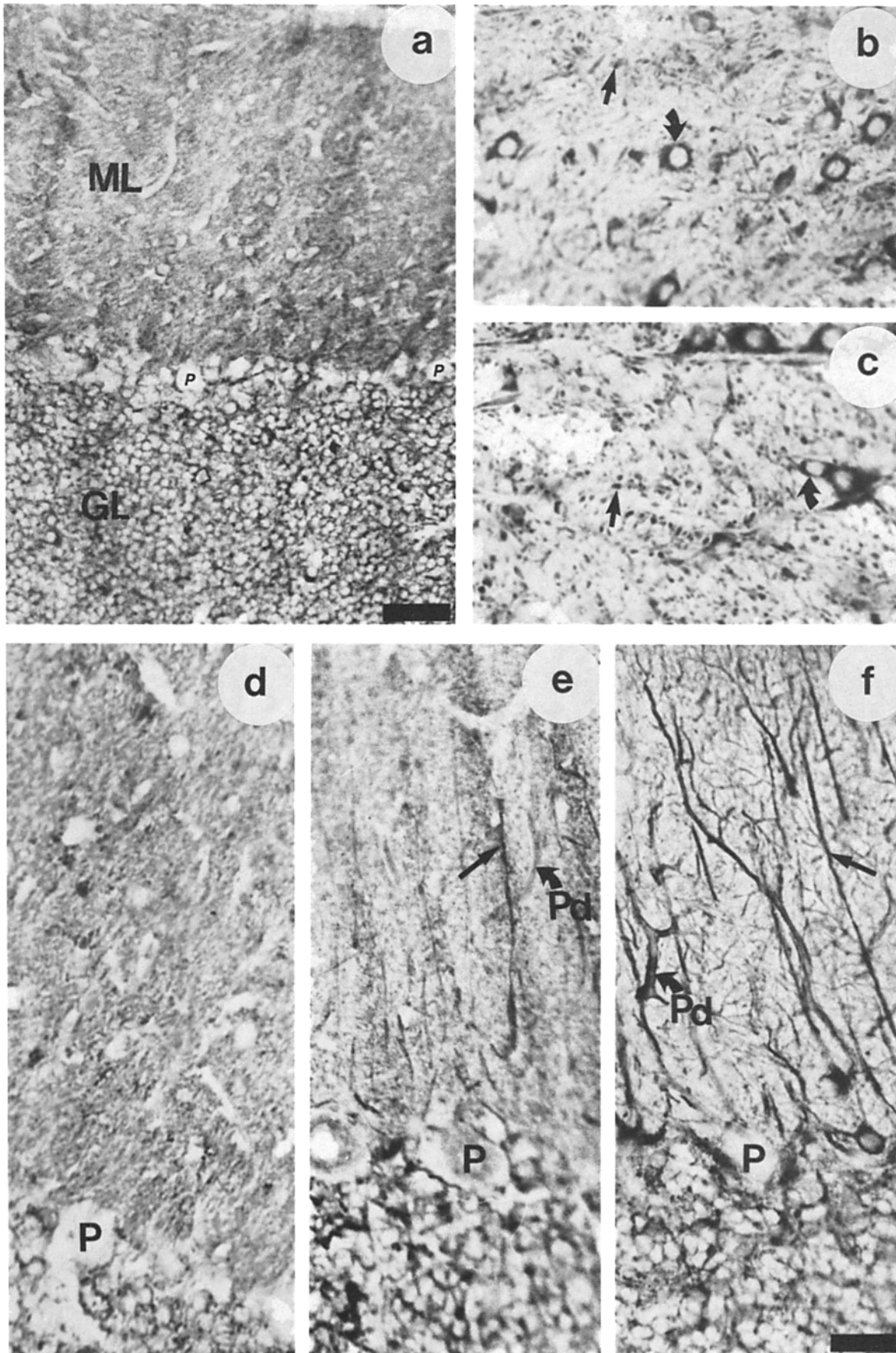
Figure 1. Immunoblotting with 6-11B-1 (acetyl-antibody) and YOL/34 (general antibody) using cerebellar homogenates. Homogenates from the cerebella of adult (*a*, *c*, and *e*) and 18-d-old (*b*, *d*, and *f*) rats were separated by polyacrylamide gel electrophoresis, transferred to nitrocellulose, and tracks incubated with 6-11B-1 (*a* and *b*), YOL/34 (*c* and *d*), or without first antibody (*e* and *f*). α -tubulin (*aT*) was stained by 6-11B-1 and YOL/34 as well as a lower molecular weight polypeptide (asterisk). Polypeptides stained nonspecifically (i.e., in the absence of first antibody) are indicated by arrowheads.

bodies within the white matter were strongly stained by the acetyl-antibody (Fig. 2 *b*); this staining pattern in white matter was indistinguishable from that with the general antibody (Fig. 2 *c*).

The staining within the molecular layer with the acetyl-antibody can be seen more clearly at higher magnification in Fig. 2 *d*. No evidence was seen in any sections of staining of Bergmann glial fibers, Purkinje cell bodies, or Purkinje cell dendrites. In contrast, the general antibody stained the Bergmann glial fibers traversing the molecular layer, and the cell bodies and dendrites of Purkinje cells as well as the parallel fibers of the molecular layer (Fig. 2 *e*). As we have shown previously (13, 14), the tyr-antibody stains Bergmann glia and Purkinje cell dendrites strongly but does not stain parallel fibers (Fig. 2 *f*). The comparison of acetyl-antibody, tyr-antibody, and the general antibody staining in the molecular layer shows that parallel fiber axons are depleted in tyrosinated α -tubulin but enriched in acetylated α -tubulin.

The interpretation of the staining patterns seen with the three monoclonals was supported by studies with other antibodies. These studies also indicated that the differences in the staining patterns with the monoclonals were not fortuitous. A rabbit antiserum (anti-tyr, reference 22) against tyrosinated α -tubulin stained vibratome sections identically to the tyr-antibody (YL1/2), and a rabbit antiserum (anti-Glu, reference 22) against detyrosinated α -tubulin stained vibratome sections identically to the acetyl-antibody (data not shown). The staining pattern seen with the general anti- α -tubulin monoclonal was indistinguishable from that

Figure 2. Immunoperoxidase staining of vibratome sections of adult cerebellum. Sections were stained with 6-11B-1 (acetyl-antibody; *a*, *b*, and *d*), YOL/34 (general antibody; *c* and *e*), or YL1/2 (tyr-antibody; *f*). Intense particulate staining is present in the molecular layer (ML) with acetyl-antibody. Bergmann glial fibers (arrows) and Purkinje cell dendrites (*Pd*) are stained by the general antibody and the tyr-antibody (*f*) but not the acetyl-antibody (*d*). In the white matter, staining with the acetyl-antibody (*b*) and the general antibody (*c*) can be seen in axons in cross section (arrows) and glial cell bodies (curved arrows). P, Purkinje cell body. Bar, (*a*) 50 μm ; (*b*-*f*) 25 μm .



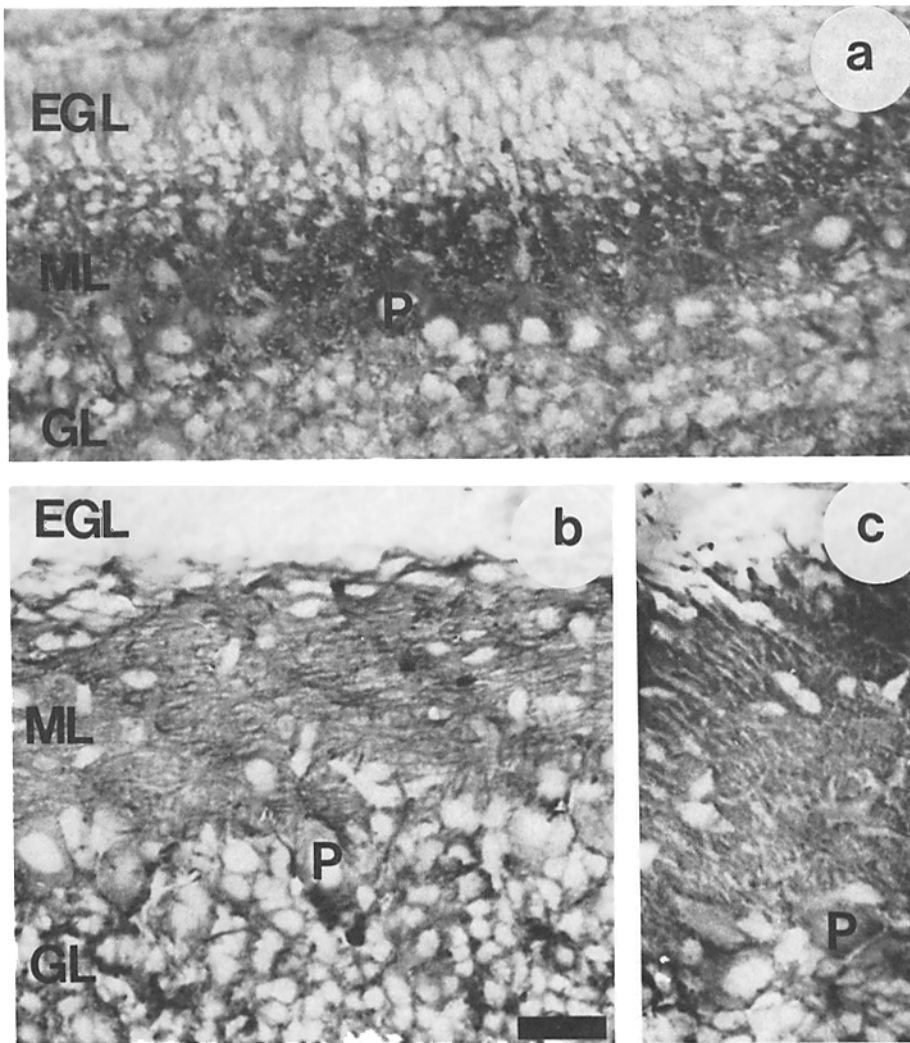


Figure 3. Immunoperoxidase staining of vibratome sections of cerebella of 7- (a) and 13- (b and c) d-old rats with acetyl-antibody (6-11B-1). Intense staining is present throughout the molecular layer (ML) at all ages with the acetyl-antibody. In b a longitudinal section through parallel fibers shows stained parallel fibers as streaks in the molecular layer. Cells in the external germinal layer (EGL) were lightly stained at day 7 but unstained at day 13. Cell bodies of the granular layer (GL) are stained at both ages. Purkinje cell bodies (P) are stained in sections from both ages. Bergmann glial fiber and/or Purkinje cell dendrites were stained by the acetyl-antibody. Bar, 25 μ m.

due to monoclonals against β -tubulin or a polyclonal antibody against α - and β -tubulin (13). Furthermore, the results with the anti- α -tubulin monoclonals did not depend on fixation conditions, since similar results were obtained using immersion fixation in formaldehyde, perfusion fixation in glutaraldehyde plus formaldehyde (13), and rapid freezing followed by staining of cryostat sections (our unpublished observations).

The α -tubulin of immature parallel fibers is tyrosinated until 10 d after birth, when there is a progressive detyrosination of α -tubulin in the maturing axons beginning in the lower (mature) region of the molecular layer (14). We therefore examined acetyl-antibody staining of vibratome sections of developing cerebella to see if acetylation of α -tubulin in parallel fibers is also developmentally regulated. The results in Fig. 3 show that acetylated α -tubulin was preferentially localized in parallel fibers throughout the molecular layer, even at early stages of development. There was no evidence of differential labeling of mature/immature parallel fibers at any developmental stage, unlike the case for tyrosinated α -tubulin (14). At no stage was staining of Bergmann glia or Purkinje cell dendrites by the acetyl-antibody detectable within the intense parallel fiber staining. The interpretation that the particulate staining in the molecular layer was due

to parallel fibers in cross section was supported by Fig. 3, b and c. Fig. 3 c shows a saggital section of 13-d-old cerebellum with parallel fibers cut in cross section. Fig. 3 b shows a section in which the parallel fibers were sectioned longitudinally and can be seen as streaks in the molecular layer.

Other Brain Regions

Studies on the cerebral cortex, corpus callosum, and brain stem indicated an enrichment of acetylated and detyrosinated α -tubulin in axons in these brain regions. Axons were relatively unstained by the tyr-antibody but intensely stained by the acetyl-antibody (data not shown), indicating that the results documented here are not peculiar to axons of the cerebellum.

Immunofluorescent Staining of Cerebellar Granule Cells in Primary Culture

Primary cultures of cells dissociated from the immature cerebellum have been extensively characterized (15) and shown to consist predominantly of granule cells and a small percentage of morphologically distinguishable nonneuronal cells. The granule cells extend processes in culture which are axonal in nature since they have synaptic-vesicle-containing

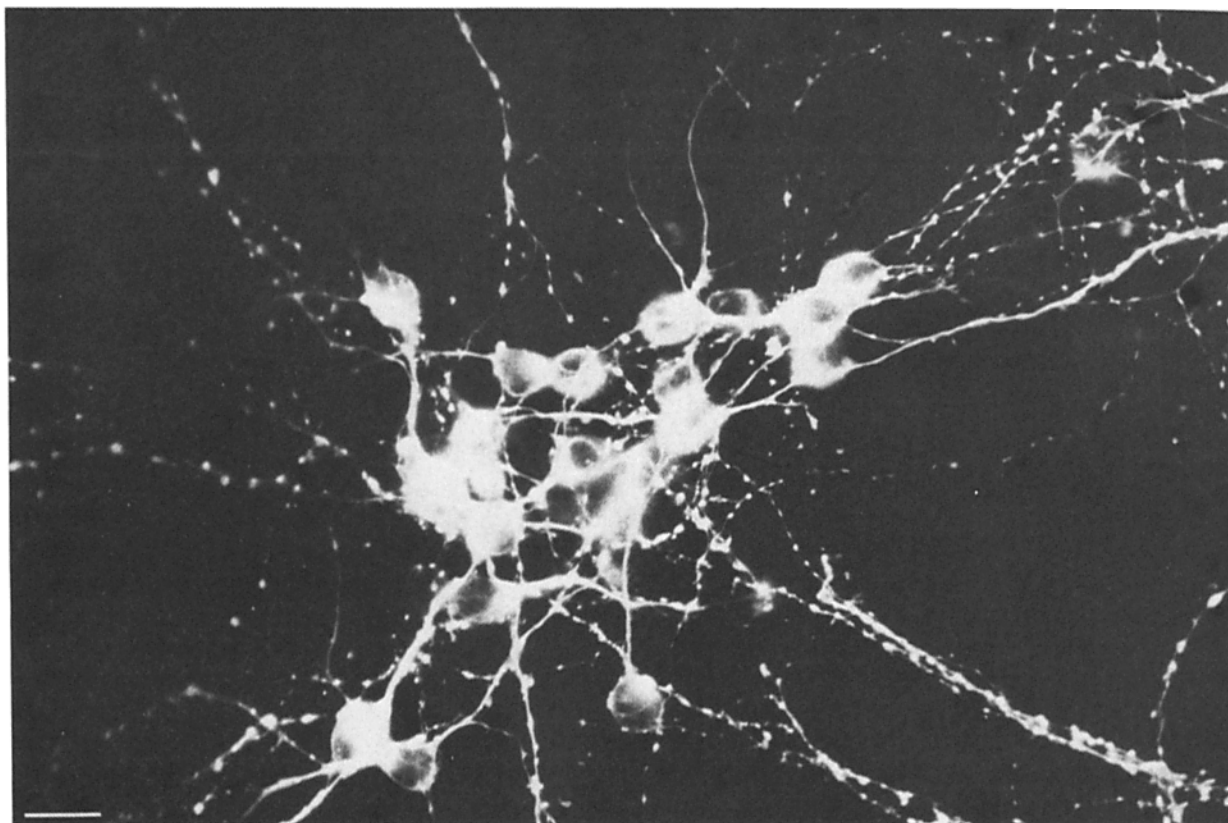


Figure 4. Immunofluorescent staining of granule cell cultures with the acetyl-antibody (6-11B-1). All granule cells in culture are stained with the acetyl-antibody throughout their cell bodies and all their axonal processes. In the processes intense varicose staining is visible. Bar, 10 μm .

varicosities (10). In cerebellar cultures it was found that the acetyl-antibody stained the cell bodies and varicose processes of all cells (Fig. 4). Acetylated α -tubulin was present, therefore, in granule cell axons in vitro as well as in vivo.

Discussion

Little is known about either the distribution or physiological roles of the various isotypic and posttranslationally modified forms of α - and β -tubulin in developing and differentiated brain tissue (6). We have previously demonstrated that axons in adult brain, particularly those that are unmyelinated, are preferentially enriched in the detyrosinated form of α -tubulin (13) as shown by lack of staining with the tyr-antibody (YL1/2) and intense staining by rabbit anti-Glu. The segregation of tyrosinated and detyrosinated α -tubulins to separate sets of microtubules has been shown in dividing and interphase cells in culture (21, 22). A monoclonal antibody against α -tubulin, TU 01, has been shown to stain only microtubules of Bergmann glia in the cerebellum (23).

Acetylation of tubulin on the ϵ -amino group of lysine was first demonstrated as a posttranslational modification of flagellar α -tubulin of *Chlamydomonas* (29). After the production of a monoclonal antibody specific for the acetylated form of α -tubulin by Piperno and Fuller (31), this form of α -tubulin was shown to be present in the axonemal microtubules from a number of sources and in stable cytoplasmic microtubules of *Chlamydomonas* (28). An activity able to

acetylate α -tubulin has been shown to be present in calf brain microtubules (20). However, the present report is the first to demonstrate the presence and localization of acetylated α -tubulin in mammalian brain.

Acetylated α -tubulin appears to be co-distributed with detyrosinated α -tubulin, both forms being predominantly present in parallel fiber axons in adult cerebellum. Axonal microtubules have been demonstrated to be relatively stable (9) and axons contain high levels of cold-stable microtubules (5). It appears then that the stable population of microtubules within axons is both acetylated and detyrosinated. The appearance of these two posttranslational modifications on stable microtubules may result from progressive modification over time of a class of microtubules with a low rate of turnover (26), or conceivably the modifications themselves may result in increased stability of microtubules. The presence of acetylated α -tubulin in many flagellar microtubules (31) and in drug-resistant microtubules of *Chlamydomonas* (28) would support a role for acetylation in the stabilization of microtubule networks as suggested by Le Dizet and Piperno (28).

There are two situations where acetylated α -tubulin is not co-distributed with detyrosinated α -tubulin. First, axons of the cerebellar white matter are strongly stained by the acetyl-antibody and are also weakly stained by the tyr-antibody (12) and so do contain some tyrosinated α -tubulin. Secondly, immature parallel fibers of the 10-d-old cerebellum are stained by the tyr-antibody (14), indicating a high content of tyrosi-

nated α -tubulin. We have demonstrated here that these immature axons also contain relatively high levels of acetylated α -tubulin.

In conclusion, results from the use of acetylated α -tubulin-specific monoclonal 6-11B-1 indicate that acetylated α -tubulin is present in certain populations of stable microtubules, including axonal, drug-stable (28), and axonemal microtubules (31). Axonal microtubules also contain the detyrosinated form of α -tubulin, suggesting that these two posttranslational modifications may have a role in the specification and/or maintenance of stable microtubule networks.

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