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The Differential Distribution of Beta Tubulin mRNAs in Individual Mammalian Brain Cells

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It has been shown by in vitro translation of polyadenylated messenger RNAs (poly(A)$^+$ mRNAs) that the mRNAs encoding both alpha and beta tubulin isotypes are present at much higher relative levels in the developing rat brain than they are in the adult [1], suggesting that the requirements for tubulin subunits vary with cell type and/or with the developmental stages of a particular cell type. The postnatally developing rat cerebellum, with its readily identifiable cell populations that perform the gamut of developmental tasks, is a suitable model for analyzing specific cellular mRNA distributions during development. In this report, by in situ hybridization techniques it is shown that, by comparison to total cellular poly(A)$^+$ mRNA levels, there is relatively more of the total beta tubulin mRNAs in mitotically active external granule layer cells than in those in the internal granule layer. These results show that migration and differentiation of these granule cells is accompanied by a decrease in their beta tubulin mRNA levels relative to the levels in granule cells of the external granule cell layer. Furthermore, the relative levels of beta tubulin mRNA both in the prenatally formed Purkinje cells and the postnatally formed stellate cells are two to fourfold less than in the granule cells of the internal granule cell layer.

Key words: tubulin, mRNA, in situ hybridization, rat cerebellum, development

Tubulin is required for several neuronal functions such as the assembly of microtubules for spindle formation [2,3], the growth and maintenance of neuronal processes [4], neurosecretion [4], the maintenance of cellular structure, and possibly migration [5]. Each of these functions is performed in one or another of the cell populations in the developing cerebellum of the 14-day-old rat [6,7]. Specifically, the granule cells of the matrix zone of the external granular layer (EGL) are dividing; the newly formed premigratory granule cells in the mantle zone of the EGL are extending axonal processes parallel to the pial surface and are perhaps "gearing up" for migration; granule cells migrating through the molecular layer toward the internal granular layer (IGL) are elaborating their axons; postmigratory basket and stellate

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cells are forming extensive dendritic arborizations that contact parallel axons of granule cells and axonal collaterals which terminate on dendrites and somata of Purkinje cells; the prenatally formed Purkinje cells are elaborating a vast dendritic network in the molecular layer; and the mature granule cells in the IGL are fully functional interneurons that are performing their differentiated tasks [5].

Our results [1,8] and those of others [9,10] show that the relative amounts of mRNAs encoding the major tubulin isotypes decrease during brain development. These results could be a consequence either of tubulin mRNA levels decreasing similarly in each cell type or of differential changes occurring in particular cell populations. The only way to differentiate between these possibilities is to use the technique of in situ hybridization to quantitate the relative levels of tubulin mRNAs in individual cell types at different stages of brain development.

In situ hybridization has been used successfully to localize the pituitary cells that synthesize proopiomelanocortin [11] and the pancreatic cells which synthesize preproelastase [12], as well as to study the induction of specific mRNAs during cell differentiation [13–16]. The specificity of this technique now has been documented in a number of systems [17–21], and several studies have shown it to be quantitative [13,18–22]. In this paper, the relative levels of the mRNAs encoding the beta tubulins are compared with total poly(A)$^+$ mRNAs in the various cell types of 14-day-old rat cerebellum. Plasmids containing chick beta tubulin DNA inserts [obtained from D. Cleveland, 23] were nick translated and the radiolabeled DNA probes were in situ hybridized to their complementary mRNAs within cells in sections of cerebella from 14-day-old rats. The distribution of the mRNAs was estimated by counting autoradiographic grains over individual cells of the various types and comparing the distribution of poly(A)$^+$ mRNA within each cell type by hybridization of [^{3}H]-polyuridylate (poly(U)) to adjacent tissue sections.

METHODS

Animals

In these experiments, 14-day-old Fischer rats were used.

Fixation and Embedding of Cerebellar Tissue

The vermis of the cerebellum was dissected free and fixed by immersion in Bouin's solution for 4 hr, then cleared with 70% ethanol. The tissue then was dehydrated through alcohols and xylenes and embedded in paraffin.

Sectioning and Slide Preparation

Sections were cut on a rotary microtome at 5 μm and collected on gelatin-chromalum subbed slides. Sections were deparaffinized in three changes of toluene and rehydrated through alcohols to water.

Preparation of Radioactively-Labeled DNA

Plasmid pBR322 with a beta tubulin insert and native pBR322 were radioactively labeled using a BRL nick translation kit and [^{3}H]-dCTP as the labeled nucleotide (59.2 Ci/mmole; NEN) to a specific activity of 6×10^5 cpm/μg and 3×10^6 cpm/μg DNA, respectively [24].

In Situ Hybridization of cDNA Probes

Either the nick-translated pBR322 probe or the beta tubulin probe was added to the hybridization buffer (10 mM Tris HCl, pH 7.6; 200 mM NaCl, and 5 mM $MgCl_2$), containing 500 μg/ml polyadenylate, 500 μg/ml sheared E coli DNA, and 25% formamide. The mixture was boiled 60 sec, quickly cooled, and then applied to the cerebellar sections (10 μl/slide; 6×10^4 cpm; 100 ng beta tubulin and 20 ng pBR322). The sections then were incubated in a moist chamber at 50°C for 1 hr, after which they were washed for 1 hr at 50°C in hybridization buffer, washed overnight in post-hybridization buffer (50 mM Tris HCl, pH 7.6, 10 mM KCl, 1 mM $MgCl_2$) at 4°C, dipped in distilled water, kept in ice cold 5% trichloracetic acid for 15 min, rinsed in distilled water and dehydrated through alcohols.

Autoradiography

Slides were dipped in NTB-2 Kodak emulsion and exposed for 5 days. Exposed slides were developed in Kodak D-19 and stained with hematoxylin and eosin y [25].

[^{3}H]-poly(U) Hybridization

Ten μl of hybridization buffer containing [^{3}H]-poly(U) (3 Ci/mmole, 4×10^4 cpm, 20 ng) was applied to cerebellar sections and incubated for 1 hr at 50°C in a moist chamber [13]. After hybridization, the slides were treated as described above for the tubulin probe hybridization. Before hybridization, control slides were incubated with RNase A (100 μg/ml) in RNase buffer (5 mM Tris HCl; pH 7.6; 1 mM KCl; 0.1 mM $MgCl_2$) for 1 hr at 37°C to degrade RNA, washed for 10 min under running tap water, and then were hybridized and treated as above [13,23].

Estimation of mRNA Content in Each Cell Type

Slides were examined at 400 to 1000 diameters and the autoradiographic grains per cell in microscopic fields, both over and under focused, were enumerated (grains above no less than 100 cells of each type were counted in cerebellar sections from 3 different animals in separate experiments). The average number of grains in each cell type in the pBR322 control sections were subtracted from the grains per cell in the sections hybridized to the beta tubulin DNA probe. Similarly, grains in the RNase-pretreated sections were subtracted from those in the sections which had been hybridized to poly(U).

Calculation of tubulin to poly(A)$^+$ mRNA ratios

The following equation was used to calculate the ratio of tubulin mRNA to total poly(A)$^+$ mRNA:

$$\frac{\text{Mean grains/cell type after tubulin cDNA hybridization}}{\text{Mean grains/cell type after poly(U) hybridization}}$$

RESULTS

In this study, the hypothesis was addressed that a cell type performing a developmental task contains relatively higher levels of specific mRNAs encoding

proteins involved in that task. In particular, the distribution of beta tubulin mRNAs was determined relative to total poly(A)$^+$ mRNAs among the different cell types and stages of granule cell differentiation in the 14-day rat cerebellum.

We used in situ hybridization of [^{3}H] poly(U) to quantitate total poly(A) levels in each cell type [11,12,21,25,26]. Conditions of hybridization were such that the poly(U) was present in 100-fold excess. Each 5 μm section of 14-day rat cerebellum contains 5 ng of total poly(A)$^+$ mRNA, and assuming an average steady state poly(A) tail size of 80 nucleotides [27], each section contains 0.2 ng of poly(A). Therefore, 20 ng of [^{3}H] poly(U) were applied to each section. Similarly, the beta tubulin probe also was present in 100-fold excess, assuming beta tubulin mRNAs comprise 10% of total mRNAs in the 14-day cerebellum.

Figures 1 and 2A, B, and C show that the majority of grains for both the poly(U) and the beta tubulin probes were localized over cells. Few, if any, grains were seen over glomeruli in the IGL, as would be expected, as glomeruli contain axon terminals [7] and hence should contain no mRNA. Two controls were performed to show that hybridization was specific. First, poly(U) did not hybridize to sections pretreated with RNase A (Fig. 1D) and RNase T2 (results not shown) demonstrating that the poly(U) was indeed hybridizing to poly(A) regions in intracellular RNA. Second, few grains were seen over sections hybridized to a control pBR322 probe (Fig. 2D), demonstrating that the tubulin DNA insert was hybridizing specifically to complementary sequences in the sections shown in Figure 2A, B, and C.

Autoradiographic grains over EGL, IGL, stellate, and Purkinje cells were counted for both the poly(U) and tubulin hybridizations. Column 1 of Table I shows that the grain counts over each cell type were highly reproducible with a standard deviation of less than 10%. Moreover, each cell type had a distinct complement of both poly(A)$^+$ mRNAs and beta tubulin mRNAs. In neither case were grain yields optimized for by, eg, varying the time and temperature of hybridization. Thus, the percentage of total poly(A)$^+$ mRNA which is tubulin mRNA cannot be calculated in each cell type. However, others have shown that the ratio of grain yields in different cell types is independent of the absolute extent of hybridization [19,28], thus allowing us to compare relative poly(A)$^+$ and beta tubulin mRNA levels even if our hybridization conditions are not yet optimal.

It was found that the stellate cells had the highest level of poly(A)$^+$ mRNA, there being an average of 70.6 grains over these cells in 5 μm sections. Purkinje cells contained an average of 30.1 grains per cell and granule cells in the EGL contained 23.0 and in the IGL, 24.3 grains per cell. These results show that the relative amounts of poly(A)$^+$ mRNA in individual cell types is not directly proportional to cell size. For example, the average granule cell in a 5 μm section has approximately the same amount of poly(A)$^+$ mRNA as does the average Purkinje cell, although the average

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Fig. 1. Autoradiographs of in situ hybridization of ^{3}H-poly(U). ^{3}H-poly(U) (3 Ci/mmole; 40,000 cpm/section) was hybridized to cerebellar sections from 14-day-old rats. A) Grain density over granule cells in the EGL. B) Grain density in Purkinje cells (P) as well as in migrating cells (M), stellate cells (S), and basket (B) cells in the molecular layer (MOL). C) Grain density in granule cells of the IGL. Note the paucity of grains over the mossy fiber axon terminals in the glomerulus (G). D) Grain density in Purkinje cells, migrating cells, stellate and basket cells, as well as in granule cells (g) of the IGL. The low density of grains over cells in D are due to pretreatment with RNAse A before hybridizations. Autoradiographic exposure was for five days. Magnification, $\times$500.

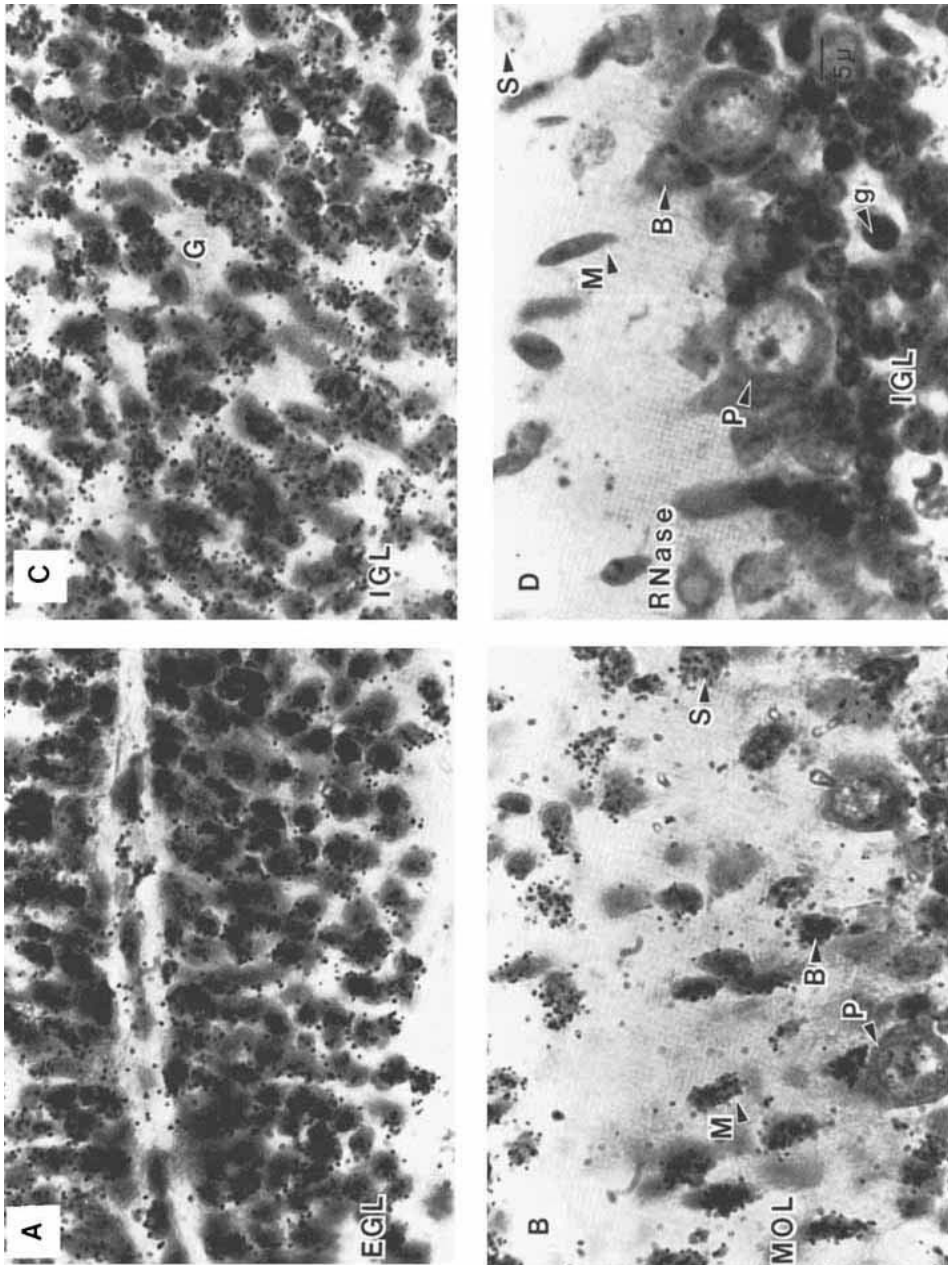

Fig. 1.

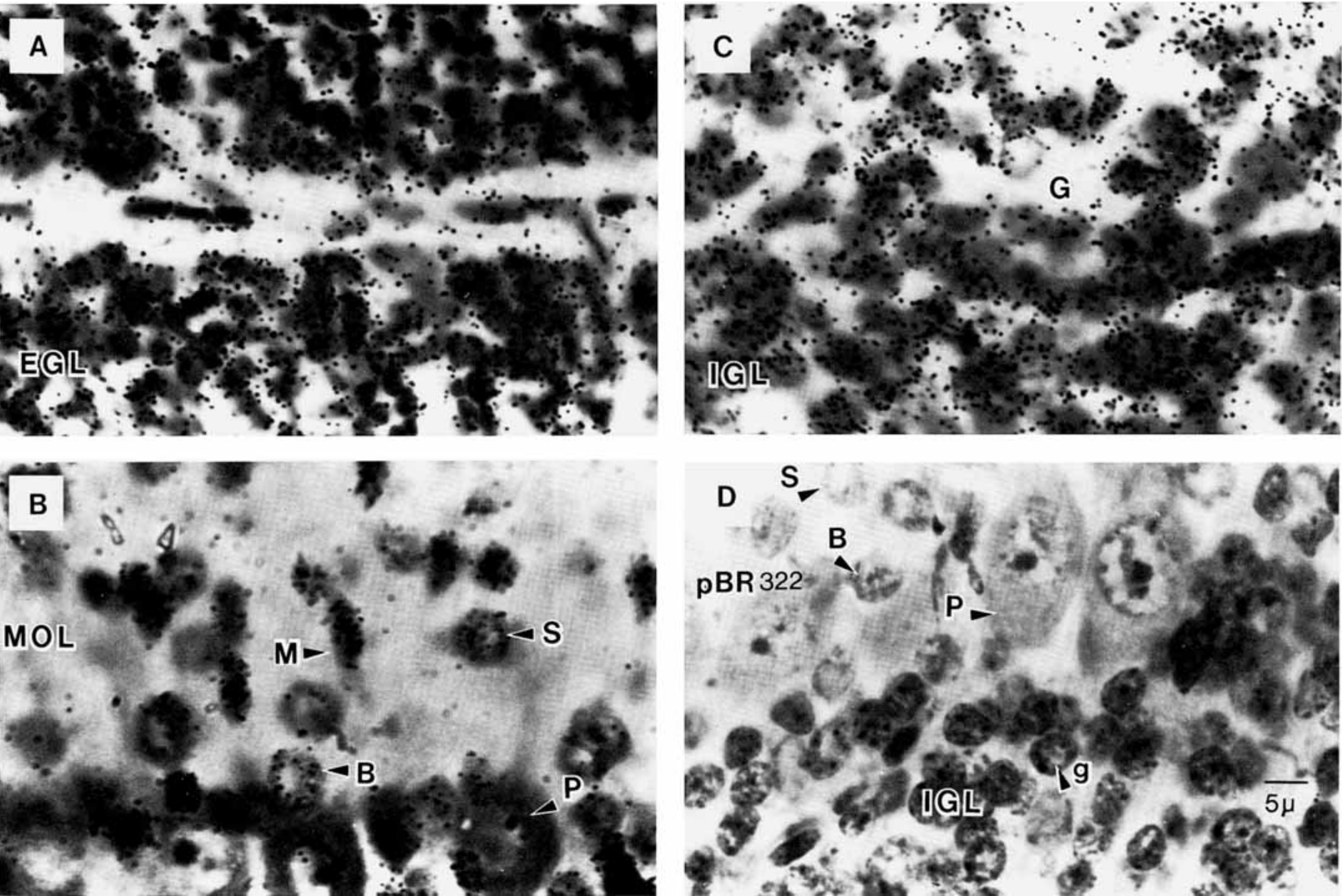

Fig. 2.

TABLE I. In Situ Hybridization of Beta-Tubulin cDNA and [^{3}H]-Poly(U) to 14-Day-Cerebellar Neurons

	Grains per cell	Relative levels of beta-tubulin mRNA
Purkinje cells		
beta-tubulin	14.7 ± 0.3	0.48
poly(U)	30.1 ± 3.4	
Stellate cells		
beta-tubulin	24.7 ± 1.8	0.35
poly(U)	70.6 ± 2.6	
EGL granule cells		
beta-tubulin	53.0 ± 2.0	2.30
poly(U)	23.0 ± 2.4	
IGL granule cells		
beta-tubulin	33.0 ± 2.4	1.36
poly(U)	24.0 ± 3.0	

The distribution of relative levels of tubulin mRNAs and total poly(A)$^+$ mRNAs over Purkinje and stellate cells and the granule cells in the EGL and IGL. Grains were counted over cells such as those shown in Figures 1 and 2. The 5 μm cerebellar sections, examined at 1000 diameters magnification, yielded "grains/cell" (mean ± SD of at least 100 cells of each cell type from three different experiments). Grains were counted by focusing at every level of the emulsion coated slide. The relative level of tubulin mRNA in each cell type is the ratio of grains/cell type in sections hybridized to the ^{3}H-beta tubulin probe to the grains/cell type in sections hybridized to ^{3}H poly(U).

cytoplasmic volume of Purkinje cells is several hundred-fold greater than that of granule cells [7].

In contrast to the cellular contents of total poly(A)$^+$ mRNA, tubulin mRNA levels were by far the highest in granule cells of the EGL (53 grains per cell). Granule cells in the IGL had an average of 33 grains per cell and stellate and Purkinje cells averaged 24.7 and 14.7 grains per cell, respectively. When the ratios of grains hybridizing to poly(A)$^+$ mRNAs and tubulin mRNAs were calculated, it was clear that the relative amount of beta tubulin mRNA was twofold greater in granule cells of the EGL compared to those in the IGL. Moreover, the relative beta tubulin mRNA content of Purkinje cells was 50% less than that of granule cells of the IGL and stellate cells contained only 25% as much.

Fig. 2. Autoradiographs of in situ hybridization of ^{3}H-beta tubulin probe and ^{3}H-native pBR322. ^{3}H beta-tubulin probe (6×10^5 cpm/μg; 60,000 cpm/section) (A–C) and native pBR322 (3×10^6 cpm/μg; 60,000 cpm/section) (D) were hybridized to cerebellar sections from 14-day-old rats. A) Grain density over granule cells in the EGL. B) Grain density in Purkinje cells (P), migrating cells (M) as well as in the stellate (S) and basket (B) cells in the molecular layer. C) Grain density in granule cells of the IGL. D) Grain density in Purkinje cells, stellate and basket cells, as well as in granule cells of the IGL. The low density of grains over cells in D shows the lack of hybridization of radiolabeled native pBR322 to cerebellar cells. Magnification, ×500.

DISCUSSION

The reproducibility of grain counts in individual cell types in these experiments clearly shows that in situ hybridization can be used quantitatively. Others have documented that, even under subsaturating conditions, grain intensity is proportional to RNA content [13,18–22]. However, it is important to note that one cannot exclude the possibility that some cells are affected differentially by tissue processing so that loss of RNA might vary according to cell type. Furthermore, the accessibility of RNAs to the probe might be different in one cell type compared to another. If either or both of these possibilities is true, comparison of the levels of poly(A)$^+$ mRNAs or tubulin mRNAs in different cell types would not be valid. However, in the likely event that poly(A)$^+$ and tubulin mRNAs are equally accessible for in situ hybridization in a single cell type, the amount of tubulin mRNA relative to the amount of poly(A)$^+$ mRNA can be compared in different cell types.

It has been assumed from morphological studies that both stellate and granule cells, with their proportionally large nuclei and organelle-poor cytoplasm, are relatively inactive compared to the Purkinje cells, with their extensive, polysome-rich cytoplasm [7]. However, if total grain counts in the poly(U) sections are an accurate reflection of intracellular poly(A)$^+$ mRNA levels in vivo, then, despite their small size (5–10 μm) [7], stellate cells contain more poly(A)$^+$ mRNA than any of the other neuronal types in 14-day cerebellum. The large Purkinje cells (21 μm) [7] contain much less poly(A)$^+$ mRNA, indeed, approximately the same amount as the small granule cells with their scant cytoplasm [7]. When the volume of cytoplasm per cell is considered, the present results therefore would suggest that the poly(A)$^+$ mRNA concentration in Purkinje cells is many fold less than in either of the other cell types. Preliminary data indicate that the paucity of mRNA in the prenatally formed Purkinje cells reflects their relative maturity at postnatal day 14, whereas the EGL and IGL granule cells have equivalent poly(A)$^+$ mRNA levels at different stages of maturity (unpublished observation). However, variations in the efficiency of hybridization to different cell types would preclude estimation of intracellular mRNA levels. Different fixation and hybridization paradigms are now being compared to determine the optimal conditions for comparison of absolute levels of intracellular mRNAs in the different cell types. A comparison of cellular mRNA levels at different developmental stages then will be necessary to determine whether mRNA levels decrease in individual cell types during development.

The distribution of beta tubulin mRNAs in the cerebellum is different from that of poly(A)+ mRNA, being highest in EGL cells. When the beta tubulin mRNA levels relative to the total poly(A)$^+$ mRNAs levels are compared (thus eliminating possible differences in the efficiency of in situ hybridization and/or differential mRNA leakage from the various cell types, as discussed above), it was found that granule cells in the EGL contain twofold more beta tubulin mRNA than those in the IGL. This could be a partial explanation for the developmental decrease in total beta tubulin mRNA levels that have been observed in both rat [1,9,10] and human [8]. The functional significance of this decrease may be related to the different metabolic requirements of granule cells during mitosis and axon formation in the EGL and during their final maturation in the IGL. In other systems, it has been shown that axon regrowth is accompanied by an increase in tubulin mRNA levels [29], indicating that normal axon growth, particularly in parallel fibers that contain microtubules and

no neurofilaments [7], may require similarly increased synthesis of tubulin. Relatively high levels of tubulin mRNAs in the premigratory cell populations of the EGL would thus be expected.

Comparison of the relative levels of tubulin mRNAs in the postmigratory, differentiated IGL granule cells, stellate and Purkinje cells shows three- to fourfold greater levels in the granule cells. This suggests that elaboration of dendrites in the 14-day stellate and Purkinje cells requires less tubulin synthesis than does maintenance of the long parallel fibers by the small cell bodies of the granule cells.

The beta tubulin recombinant DNA probe used in this study is a full-length copy of a chick beta tubulin mRNA [23,30] and thus will hybridize with the coding regions of the mRNAs for the other beta tubulin isotypes [31,32]. However, under our stringent experimental conditions, it is unlikely that the probe hybridizes to any non-beta-tubulin mRNAs. Several lines of evidence indicate that a number of beta tubulin mRNAs are expressed in vertebrate cells [1–8,10,31,32], but only one, the beta 1 subunit, is decreased dramatically during brain development [1,8]. Subcloning of the 3′ ends of the rat beta tubulin mRNAs from our rat cerebellar cDNA library [Morrison and Sparkman, manuscript in preparation] and hybridization of these probes to cerebellar slices should allow us to determine whether the different tubulin isotypes are localized in specific cell types or whether the beta 1 tubulin mRNA is synthesized preferentially in the dividing and premigratory cells. Such an analysis would indicate a specific function for the beta 1 subunit.

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ADDENDUM

We have recently shown by Northern analysis of cerebellar mRNAs that the chick β tubulin probe encodes the same mRNA as the rat RBT.2 probe of Bond *et al* [Mol Cell Biol 4:1313, 1984] and shows little or no cross hybridization with mRNAs to the more abundant brain β tubulin mRNA which is also expressed in other tissues. Therefore, our in situ hybridization is to the brain-specific β tubulin mRNA, the levels of which decrease dramatically during development of both cerebellum and cortex [1,10].