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MAP2d mRNA is expressed in identified neuronal populations in the developing and adult rat brain and its subcellular distribution differs from that of MAP2b in hippocampal neurones

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Abstract

The brain microtubule-associated protein MAP2 family is composed of high-molecular-weight (MAP2a and MAP2b) and low-molecular-weight (MAP2c and MAP2d) isoforms. The common C-terminal region of HMW MAP2 and MAP2c contains three repeated microtubule-binding domains while MAP2d comprises four repeats. MAP2c mRNA is known to be expressed at high levels in the immature brain. We show that in the brains of rat pups, MAP2c mRNAs are indeed expressed at high levels compared with MAP2d. However, in adult rat brains, MAP2d mRNA levels are higher than MAP2c. In order to identify the neural cells expressing MAP2d, we used *in situ* hybridization. *In vivo*, we show that MAP2d mRNA is expressed in well-identified neuronal populations of the brain. In primary cultures of hippocampal neurones, double-labelling experiments confirm that MAP2d is clearly expressed in neurones. We also evaluated in this study the subcellular distribution of the MAP2d mRNAs in cultured hippocampal neurones and we report that in contrast with MAP2b mRNAs, mostly localized in dendrites, MAP2d mRNAs are essentially located in neuronal cell bodies.

Introduction

Microtubule-associated protein-2 (MAP2) is a major component of the neuronal cytoskeleton (Ludin & Matus, 1993) that exists in high-(280 kDa) and low-(65–70 kDa) molecular-weight forms, MAP2a-MAP2b and MAP2c, respectively. MAP2 variants are encoded by distinct mRNAs of 9 and 6 kb, transcribed from a single gene (Lewis *et al.*, 1986; Neve *et al.*, 1986; Garner & Matus, 1988; Doll *et al.*, 1993; Kalcheva *et al.*, 1995; Chung *et al.*, 1996). A small MAP2 transcript that we have called MAP2d (Ferhat *et al.*, 1994a), has been described recently (Doll *et al.*, 1993; Ferhat *et al.*, 1994b; Kindler & Garner, 1994) and comprises a 93-bp insertion encoding 31 additional amino acids in the carboxy terminal region when compared with MAP2c. This insertion encodes a domain highly homologous to the three microtubule binding domains found in MAP2 proteins, and to the fourth microtubule binding domain encoded by the alternatively spliced exon 10 of tau mRNA (Kosik *et al.*, 1989; Ferhat *et al.*, 1994a). In rat brain, the large MAP2 mRNAs do not contain the 93-bp exon (Doll *et al.*, 1993; Ferhat *et al.*, 1994a; Ferhat *et al.*, 1994b; Forleo *et al.*, 1996) while this is not the case in the rat dorsal root ganglia (Forleo *et al.*, 1996). Using transient cell transfections, we have shown that MAP2d promotes substantial organization and stabilization of both microtubules and microfilaments when compared with MAP2c, suggesting a definite role for MAP2d in the differentiation of neuronal cell extensions (Ferhat *et al.*, 1996a). Splicing of the MAP2d additional exon is developmentally regulated, MAP2d mRNAs being predominantly expressed over MAP2c mRNAs in various adult rat brain structures (Ferhat *et al.*, 1994b). In contrast, MAP2c is expressed at higher levels in the immature brain (Couchie & Nunez, 1985; Riederer & Matus, 1985; Ferhat *et al.*, 1994b). *In situ* hybridization shows that the mRNAs for the high-molecular-weight form of MAP2 are expressed both in cell bodies and in dendrites, whereas the MAP2c mRNA is only detected in neuronal cell bodies (reviewed in Goedert *et al.*, 1991; Marsden *et al.*, 1996). Immunohistochemistry has been used to localize the corresponding MAP2 protein isoforms in neuronal cells: the high-molecular weight forms of MAP2 are expressed exclusively in dendrites of neurones (Tucker & Matus, 1988), while MAP2c is distributed in all neuronal compartments (Meichsner *et al.*, 1993). Although we have shown that MAP2d mRNA expression is higher in primary neuronal cell cultures than in cultured glial cells (Ferhat *et al.*, 1994b), Doll *et al.*, 1993) find increased levels of the four repeat MAP2 protein in glial cell cultures when compared with neurones. Because little is known about MAP2d expression at the cellular level during cerebral ontogeny, and in the absence of MAP2d-specific antibodies, we have addressed this issue *in vivo* using *in situ* hybridization.

We describe the regional expression and cellular localization of mRNAs encoding MAP2d in the developing and adult rat brain. We

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show that MAP2d mRNAs are clearly expressed in well identified neuronal populations in CNS structures that include cerebellum, olfactory bulb and hippocampus. Using double-labelling techniques, we confirm in cultured hippocampal cells that MAP2d is expressed in neurones. Finally, in contrast to MAP2b mRNAs which are present in dendrites, we show that the MAP2d mRNAs are located mainly in neuronal cell bodies.

Materials and methods

RNA extraction, RT-PCR and analysis of amplified DNA products

Wistar rat pups ranging from birth (P0) to 21 days of age (P21) (three–four animals per age-group) or adult (2 months old, two animals per age group), were killed and the brains were rapidly removed for RNA extraction. Total RNAs from rat brains at various postnatal days (P0, P4, P7, P10, P14, P21 and adult) were extracted as described in Ferhat *et al.* (1994b).

In order to follow the expression of MAP2c and MAP2d in the developing brains, semiquantitative reverse transcription polymerase chain reaction (RT-PCR) was performed using primers specific for MAP2: primer 1, CACAAGGATCAGCCTGCAGCTCTG; primer 2, CACCTGGCCTGTGACGGATGTTCT; nested probe 3, CAATCCTCCCTCCTCGCAGGGGCG. Primers used in this study were selected in the rat MAP2d sequence and their respective positions are 204–235; 1323–1353 and 723–758 (Ferhat *et al.*, 1994a). We also used an exogenous *in vitro* transcribed RNA encoding tobacco nitrate reductase (NR) as a standard. Briefly, equal amounts (1 μg) of total RNAs extracted from the rat brains were spiked with 40 pg of NR RNA. cDNA synthesis, PCR reactions (with the following profile: 15 s denaturation at 94 °C, 30 s annealing at 55 °C, 60 s elongation at 72 °C), gel analysis in 1% agarose gels, southern blots and hybridizations with a γ-^{32}P-labelled nested probe were performed as described in Ferhat *et al.* (1993). The blots were rinsed in 2 × standard saline citrate (SSC) at room temperature and washed in 2 × SSC, 1% sodium dodecyl sulphate (SDS) at 60 °C for 30 min. Hybridized membranes were exposed to Amersham MP films from 12 h to 4 days. For each cDNA, three PCR reactions were performed (26, 28, 30 cycles) to guarantee that the results are obtained in the linear phase of the amplification process. Identical results were obtained from two independent PCR experiments. The validity of the PCR assay was assessed by performing PCR using ratios of MAP2d/MAP2c plasmids that varied from 0/10 to 10/0 in unit increments (Ferhat *et al.*, 1994b).

Hippocampal cell cultures

Pregnant Wistar rats were killed on gestational day 18 for collection of the embryos. The hippocampi were dissected under sterile conditions in phosphate-buffered saline (PBS) containing 0.6% (wt/vol.) glucose. Tissues were incubated for 20 min at room temperature in calcium-magnesium-free Hank's balanced salt solution (GIBCO) containing 0.1% trypsin (GIBCO) and 0.1% (wt/vol.) DNAse (type I, Sigma). The reaction was stopped with 1% (wt/vol.) soybean trypsin inhibitor (Sigma) and the tissues were mechanically dissociated through a fire-narrowed polished Pasteur pipette in minimal essential medium (MEM) (GIBCO BRL) supplemented with 0.05% DNAse. After a brief centrifugation the cell pellets were resuspended in the culture medium (see below) and the cells were counted.

For *in situ* hybridization and immunohistochemistry, cells were plated on glass coverslips (18 mm) which had been previously coated with 50 μg/mL of poly-L-lysine (MW 30 000–70 000; Sigma) from a stock solution in 0.1 M borate buffer, pH 8.3. Cells were cultured at low density on glass coverslips to allow morphological analysis, in minimal medium consisting of MEM (containing 20 g/L glucose), the N2 supplements according to Bottenstein & Sato (1979) and 0.1% (wt/vol.) bovine serum albumin without serum, eventually in the presence of human recombinant basic fibroblast growth factor (bFGF) (PBH, Hanover, Germany; 25 ng/mL) for 2 days, to induce MAP2d expression (Khrestchatisky *et al.*, 1995).

In situ *hybridization using ^{35}S-dATP radiolabelled probes*

The sequences of the MAP2b and MAP2d-specific oligonucleotides were, respectively: CCAAGGGCAATAGAATCAAGGCAAGACATAGGCAA (2178–2212) (Kindler *et al.*, 1990) and TATCCTTGGAACCACATCTTGACTGAACTT (1106–1077) (Ferhat *et al.*, 1994a). A Southern blot (as described in Ferhat *et al.*, 1993) with the MAP2c and MAP2d cDNAs (described in Ferhat *et al.*, 1996a) was performed to control the specificity of MAP2d *in situ* oligonucleotide probe used in this study. cDNA inserts were spliced out using *Eco*RI digestion and DNA products were separated in a 1% agarose gel. Washing conditions of the hybridized membrane were identical to those used for the *in situ* hybridization experiments. The MAP2d *in situ* probe was radiolabelled and hybridization on tissue sections was performed as described previously (Ferhat *et al.*, 1996b,c,d). Cells on glass coverslips were fixed for 15 min in 4% paraformaldehyde diluted in 0.1 M PBS, at room temperature, rinsed three times for 5 min in PBS (one time in 0.3 M; two times in 0.1 M PBS) and dehydrated in graded alcohols (30, 50, 95 and 100%). Hybridization was overnight at 42 °C with 250 μL hybridization mix containing 50% deionized formamide, 4 × SSC, 5 × Denhardt's solution, 10% dextran sulphate, 250 μg/mL yeast tRNA, 250 μg/mL salmon sperm DNA, 100 mM dithiothreitol (DTT), 250 μg/mL Poly A. 2×10^5 c.p.m. of probe were used per coverslip.

Following hybridization, tissue sections and cells on the coverslips were rinsed at room temperature in 4 × SSC, 2 × SSC (2 × 15 min) and 1 × SSC (2 × 15 min). High stringency wash was in 0.1 × SSC at 55 °C (2 × 30 min). All rinse and wash buffers contained 1 mM DTT. The slices and coverslips were dehydrated in 30, 50, 95 and 100% ethanol containing 330 mM Ammonium Acetate, and air dried. The slices were exposed to Hyperfilm β-max (Amersham) for 12 days. Some sections were then dipped in Amersham LM1 emulsion and exposed at 4 °C for 30 days. After development, sections were counterstained with cresyl violet. Controls consisted in the hybridization of adjacent sections with an excess (200 pmols) of non-radioactive probe in the hybridization solution. Identical results were obtained when using a riboprobe transcribed *in vitro* from a plasmid containing only the MAP2d specific insertion; in these experiments, high-stringency washes were 0.1 × SSC at 65 °C (2 × 30 min). The coverslips were also dipped in Amersham LM1 emulsion and exposed at 4 °C for 3 weeks. After development, coverslips were counterstained with cresyl violet. In some experiments, cells on coverslips were counterstained with a MAP2 antibody as described below prior to the emulsion. For these double-labelling experiments, hybridized coverslips were incubated overnight with the primary antibody diluted in PBS and immunoreactivity was revealed with the avidin–biotin–peroxidase system (Vector) using diaminobenzidine as a substrate as described in Ferhat *et al.* (1996c). The coverslips were rinsed and dipped in Amersham LM1 emulsion and exposed at 4 °C for 3 weeks. After development, coverslips were mounted and viewed under light microscopy. In these experiments, specificity of the hybridization signal was assessed using (i) labelled sens oligonucleotide and riboprobes and (ii) excess (200-fold) non-labelled probe added to the labelled oligonucleotide probes.

In situ *hybridization using digoxyginine labelled probes*

In situ hybridization was performed using the same MAP2b and MAP2d oligonucleotide probes described above. Twenty picomoles of each oligonucleotide were labelled in a 25 μL reaction mix containing 2.5 mmol Dig-11-dUTP (Boehringer), 30 units terminal deoxynucleotidyl transferase (TdT) (BRL) and 1 × TdT buffer (BRL). Incubation was for 1 h at 37 °C. The reaction was stopped by adding 1 μL of 0.5 M EDTA pH 8.0. The probes were purified by ethanol precipitation. Pellets were washed with 75% ethanol and resuspended in 100 μL of diethyl pyrocarbonate-treated water and stored until needed at –20 °C. Hybridization was performed as described above for the (^{35}S-dATP) labelled probes except that 2 μL of the non-radioactive probes were added to the hybridization mix. After overnight incubation, coverslips were rinsed in 4 ×, 2 × and 1 × SSC at room temperature, washed twice for 15 min in 0.5 × SSC at 55 °C. All rinse and wash buffers contained 1 mM DTT. Coverslips were then incubated in PBS containing 2% gelatine and 0.2% Triton X-100 for 20 min at 37 °C to block non-specific immunostaining. This was followed by an overnight incubation at room temperature with an anti-Dig antibody conjugated with fluorescein (FITC) (5 μg/250 μL PBS 1×, Boehringer). Finally, after washes in 1 × PBS, coverslips were mounted and viewed under fluorescence microscopy. Controls for the specificity of the labelling consisted (i) in the incubation of the coverslips in hybridization solution without probe, followed by revelation with anti-Dig antibody conjugated with fluorescein (FITC); no fluorescent signal was detected (data not shown); (ii) excess (200-fold) non-labelled probe added to the labelled probe; (iii) labelled sens oligonucleotide probes.

Results

Relative levels of MAP2c and MAP2d during rat brain development

We analysed by RT-PCR the MAP2c and MAP2d expression levels in RNAs extracted from P0, P4, P7, P10, P14, P21 and P60 rat brains (Fig. 1B). We performed PCR with primers (1–2) (Fig. 1A) and following gel analysis and Southern transfer, the specificity and relative amounts of the amplified DNA products were assessed using a labelled nested oligonucleotide probe (probe 3, Fig. 1A). RT-PCR analysis demonstrates in rat brain elevated levels of MAP2c mRNAs at P0 and until P14-P21, followed at P60 by a decrease in their steady-state levels (Fig. 1B). The lowest MAP2d mRNA levels are found at P0, followed, from P4 until P21, by an increase in the steady state levels and a decrease at P60. Figure 1(B) shows that in the course of ontogeny MAP2c/MAP2d ratios decreased. In the brain, inversion of the MAP2c/MAP2d ratio occurs between P21 and P60 when MAP2d levels decrease but are predominant over MAP2c. Intermediate size PCR products were detected between MAP2c and MAP2d at P4 to P10. They likely correspond to hybrid MAP2c-MAP2d molecules generated by renaturation because such intermediate size products are occasionally found when performing PCR with mixtures of MAP2d and MAP2c plasmids. Our attempts to clone these PCR products have lead to the cloning of either MAP2c or MAP2d PCR products. Addition of *in vitro*-transcribed tobacco NR RNA to the total RNA samples before cDNA synthesis allows standardization following cDNA synthesis, PCR reactions and transfer procedures. In these experiments, NR hybridization signals were similar in all samples (Fig. 1B).

Regional distribution of MAP2d mRNA in the developing rat brain

Because the microtubule binding domains of MAP2 share sequence homology, specificity of the *in situ* hybridization oligonucleotide probe MAP2d (Fig. 1A) was assessed. The MAP2d *in situ* hybridization probe selected in the MAP2d specific exon does not cross-hybridize with the related microtubule binding domains of the MAP2c cDNA as shown on a southern blot with MAP2c and MAP2d plasmids (Fig. 1C). *In situ* hybridization experiments were performed on representative sagittal sections of brains dissected at the embryonic stage (E18), at birth (P0), during postnatal development (P10, P14, P21) and from adult (P60) rats. MAP2d mRNA expression (Fig. 2) was low in the embryonic brain and increased throughout brain development (P0, P10, P14, P21) in CNS structures that include cerebellum, olfactory bulb, hippocampus and cortex. At P10, MAP2d labelling is clearly detected in the developing cerebellum and olfactory bulb. In the latter structure, the periglomerular layer is more strongly labelled at P10 and P14 than the granular layer. At P21 and beyond, both layers are highly labelled. At all stages of brain ontogeny, the cortex is poorly labelled when compared with the cerebellum and olfactory bulb. In the hippocampus, at all stages, the dentate gyrus is more prominently labelled than the pyramidal cell layer.

We have compared in this study the distribution of MAP2d and MAP2b mRNAs in the adult rat brain. *In situ* hybridization was performed using an oligonucleotide probe specific for MAP2b. MAP2b distribution is more homogeneous across the different brain structures than MAP2d. MAP2b mRNA is prominently expressed in granule neurones of the cerebellum and in periglomerular and granule neurones of the olfactory bulb (Fig. 2) but also in structures where MAP2d is poorly expressed such as cortex and hippocampus. In this structure, the dentate gyrus and pyramidal cell layer are strongly labelled with a diffuse labelling characteristic of dendritic localization of the mRNA.

Cellular distribution of MAP2d mRNA in the developing rat brain

Analysis of emulsioned slices allowed the identification of some of the cell types expressing the MAP2d mRNA (Figs 3, 4 and 5). In the hippocampus (Fig. 3) of P0 rat pups (Fig. 3A), high expression was seen in the cells of the molecular layer and in the cells that distribute within the hilus during this period of life. From P0 to P60, MAP2d mRNA expression was also observed in granule neurones of the dendate gyrus (Fig. 3A,B,C,D). Labelling was higher in pyramidal neurones of the hippocampus (Fig. 3E) of rat pups (P0), than in adult animals, where low levels of MAP2d expression were detected (Fig. 3F).

In the cerebellum (Fig. 4), the external and internal granule cells of the cerebellum of rat pups at P10 and P14 were highly labelled (Fig. 4A,B,C). At P10, in the molecular layer, some cells, presumably migrating granule neurones, are labelled. In the adult rats, granule neurones of the cerebellum also expressed MAP2d mRNA but the labelling was slightly reduced compared with the earlier time-points (Fig. 4D). The Purkinje cells of the adult rats were slightly or not labelled and Bergmann glial cells located between Purkinje cells were not labelled (Fig. 4D).

In the olfactory bulb (Fig. 5), MAP2d mRNA expression was higher in adults (Fig. 5B,D) than in the P10 rat pups (Fig. 5A,C). At P10, a slight expression of the MAP2d mRNA was observed in the periglomerular (Fig. 5A) and granule neurones (Fig. 5C). In adults (Fig. 5B,D), all these neurones (periglomerular and granule neurones) express high levels of MAP2d mRNA.

MAP2d expression in cultured hippocampal neurones

In order to ascertain the fact that MAP2d was expressed in neurones, we assessed MAP2d mRNA expression in primary cultures of hippocampal neurones by combining *in situ* hybridization with a ^{35}S-labelled oligonucleotide probe specific to MAP2d, with

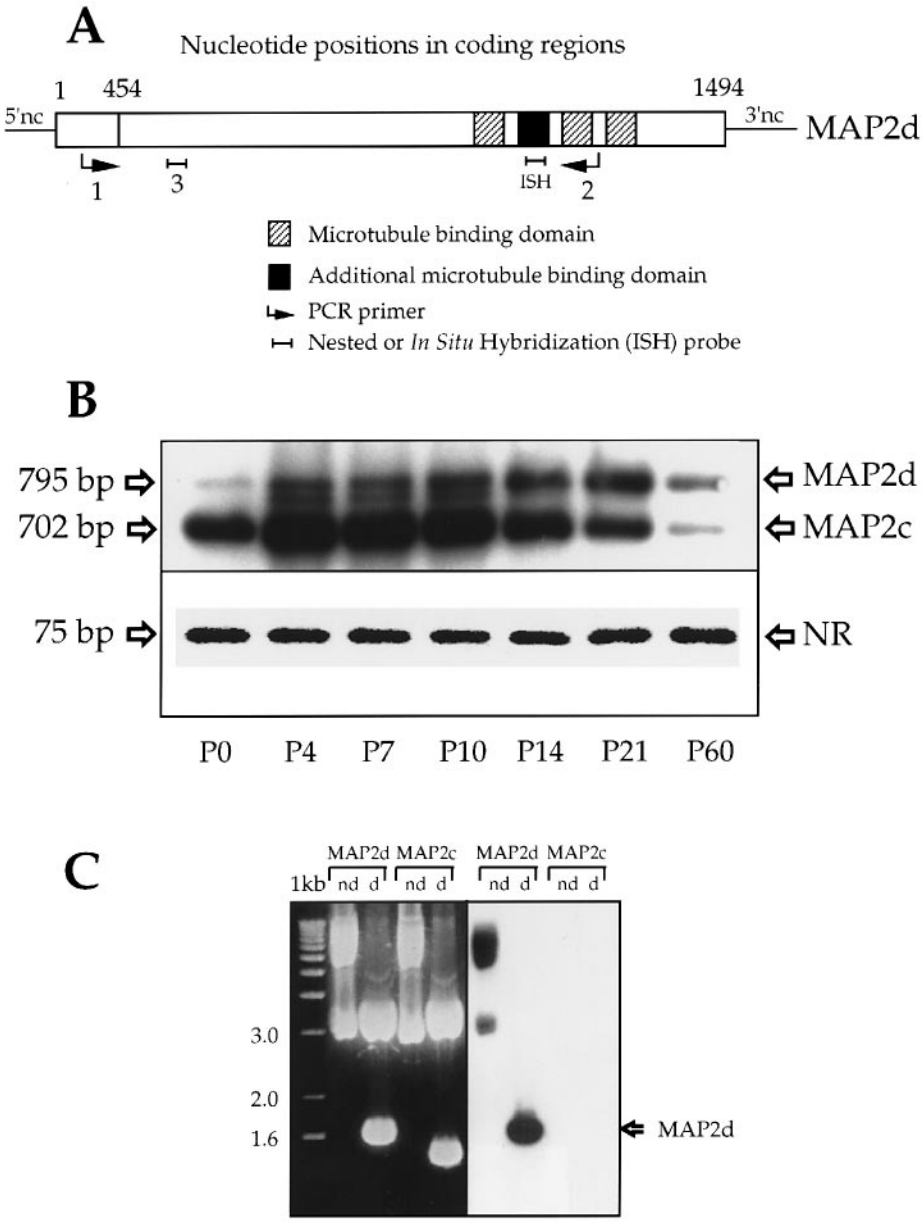

FIG. 1. (A) Schematic representation of MAP2d and relative positions of the polymerase chain reaction (PCR) primers (primers 1 and 2) and the nested (oligonucleotide 3) or *in situ* hybridization (ISH) probe used in this study. 5′ and 3′ non-coding regions: thin line. The coding region is boxed. Light hatched boxes: microtubule binding domains. Numbers refer to nucleotide positions in the coding regions of MAP2d.
(B) Relative expression of MAP2c and MAP2d mRNAs in the developing rat CNS determined by RT-PCR with primers (1–2). The MAP2c/MAP2d ratios were analysed in RNAs extracted from whole brain at P0, P4, P7, P10, P14, P21 and P60 animals. Following gel analysis and Southern transfer, membranes were hybridized with oligonucleotide 3. The MAP2c and MAP2d amplification products are, respectively, 702 and 795 bp in length. All samples underwent 30 cycles PCR. *In vitro*-transcribed tobacco nitrate reductase (NR) RNA was used as an exogenous cDNA and PCR standard. Note the inversion of the MAP2c/MAP2d ratio in the P60 sample when compared with P0. (C) Specificity of the MAP2d *in situ* oligonucleotide probe. The non-digested (nd) and *Eco*RI-digested (d) MAP2d and MAP2c plasmids were analysed in a 1% agarose gel stained with ethidium bromide. The MAP2d and MAP2c *Eco*RI-digested inserts are, respectively, 1594 and 1501 bp in length. Following southern transfer and hybridization of the membranes with MAP2d *in situ* hybridization probes, only the non-digested MAP2d plasmid and MAP2d insert are detected by the MAP2d probes.

immunocytochemistry using an antibody against the neurone specific microtubule-associated protein MAP2 (Fig. 6). We have previously shown (Khrestchatisky *et al.*, 1995) that MAP2d expression is enhanced in hippocampal neurones following bFGF stimulation (25 ng/mL). In order to increase the cellular levels of MAP2d expression, neuronal cultures were grown for 2 days in the presence of bFGF. In unstimulated conditions, MAP2d mRNAs were barely detectable in hippocampal neurones (Fig. 6A). In contrast, a strong labelling was observed when neurones are stimulated for 2 days with bFGF because silver grains were densely localized over the soma of all MAP2 immunoreactive cells (Fig. 6B).

Subcellular localization of the MAP2d and MAP2b mRNAs in cultured hippocampal neurones

In situ hybridization experiments in the developing and adult rat brains (Fig. 2) revealed important differences in the MAP2d and

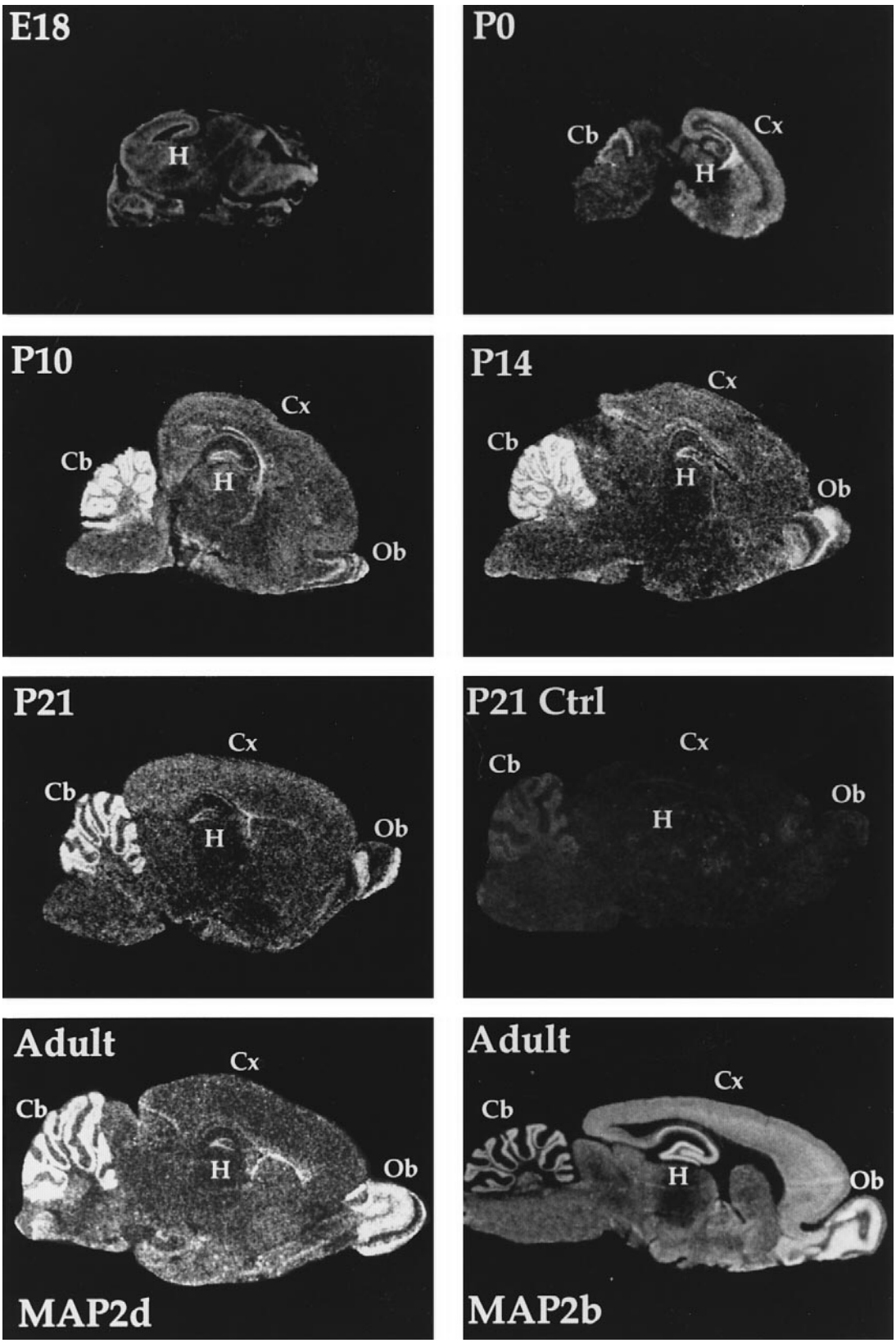

FIG. 2. Regional localization of MAP2d mRNA during rat brain development, determined by *in situ* hybridization. Autoradiographs are representative of the hybridization patterns obtained with the MAP2d probe in the brains of E18 embryos (E18), at birth (P0), and at postnatal days P10, P14, P21 and in the adult rat brain, respectively. A 200-fold excess of non-labelled oligonucleotide probe displaces labelling with the radiolabelled probe as shown on a P21 brain section (P21 Ctrl). The lower right panel corresponds to the hybridization pattern obtained with the MAP2b probe in the adult brain. Abbreviations: Cb, cerebellum; H, hippocampus; Cx, cortex; Ob, olfactory bulb.

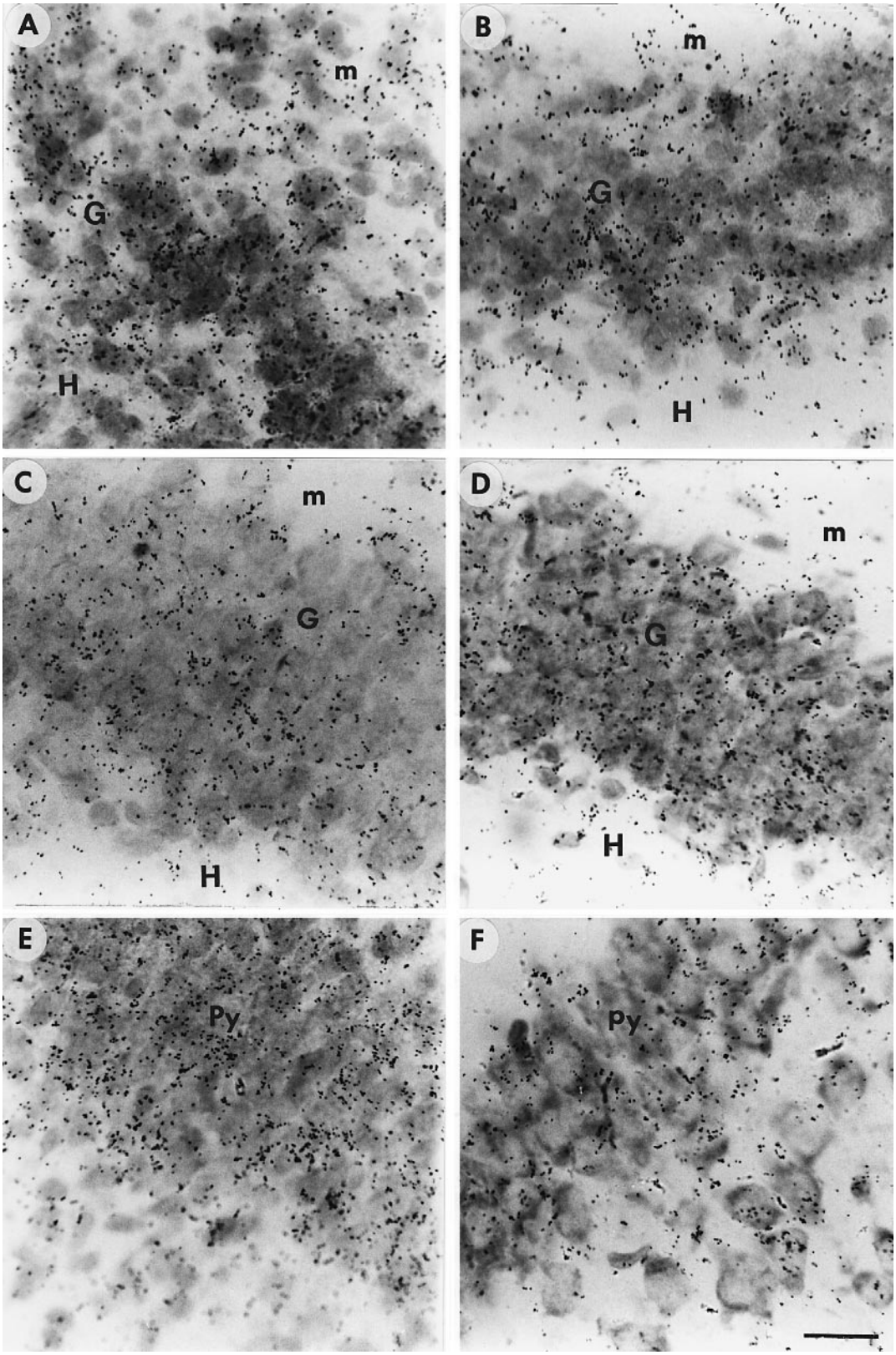

FIG. 3. Cellular localization of the MAP2d mRNAs during development of the hippocampus. (A) Saggital section showing the dentate gyrus of a P0 rat pup. Note the intense hybridization signals in the granule cells (G) of the dentate gyrus and the cells of the hilus (H). In the molecular layer (m) of the dendate gyrus, some cells are heavily stained by the MAP2d probe. (B, C and D) Saggital sections showing the dentate gyrus of P10, P14 and adult rats, respectively. In the latter, the granule cells of the dentate gyrus still express high levels of the MAP2d mRNA. (E and F) Saggital sections showing the pyramidal cell layers (Py) of a P0 and adult rat. Note that the pyramidal neurones which are already in the process of differentiation at P0, express more MAP2d at this stage than in the adult. Scale bar: 25 μm.

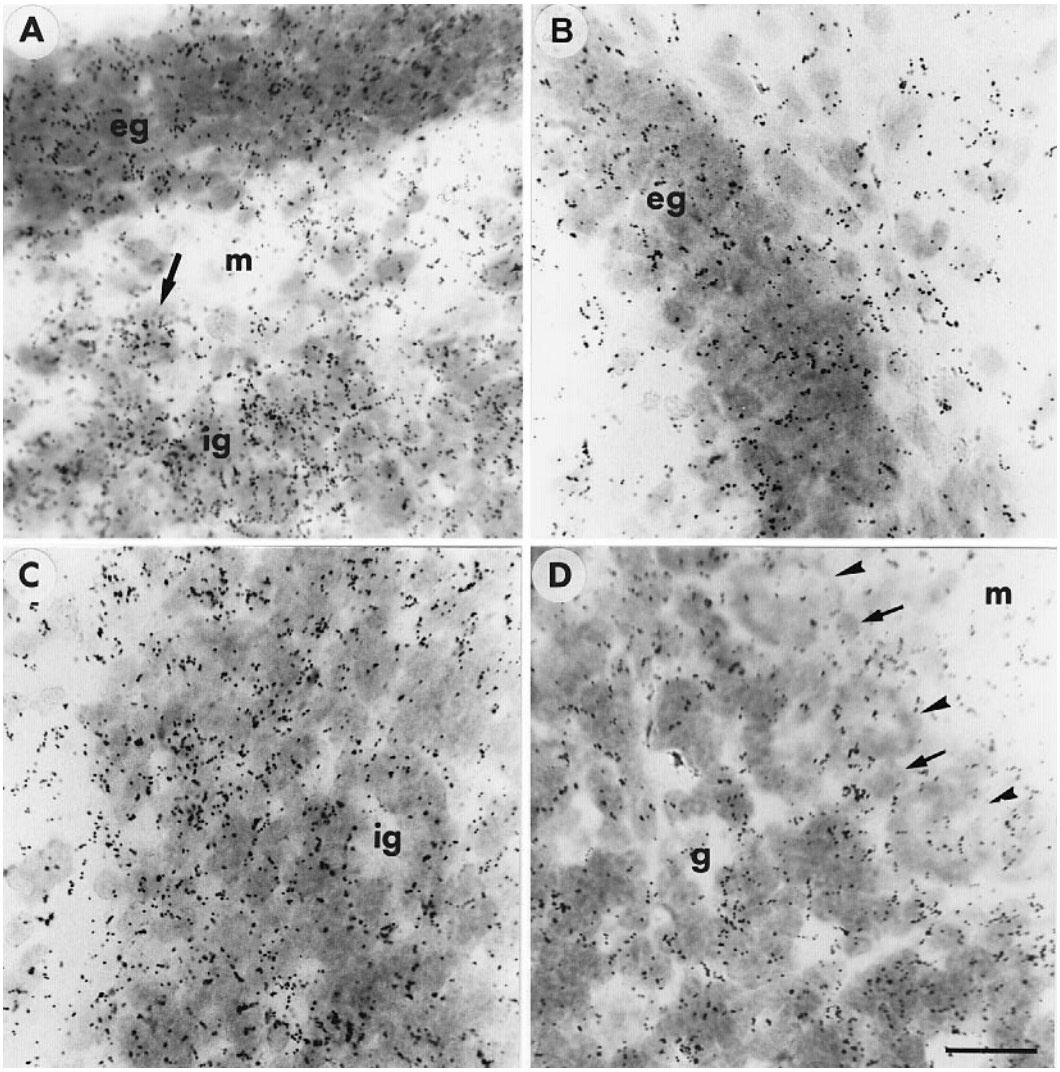

FIG. 4. Cellular localization of the MAP2d mRNAs during development of the cerebellum. Saggital sections showing the cerebellum of P10 (A), (P14) (B and C) rats. At P10 and P14, all cell layers express MAP2d mRNAs: the external granule cell layer (eg), the internal granule cell layer (ig) and the molecular cell layer (m). In (A), the arrow indicates a MAP2d labelled cell in the molecular layer, presumably a migrating neurone. (D) Saggital section of an adult cerebellum: note that the granule neurones (g) in the adult cerebellum still express MAP2d mRNAs but at lower levels than in pups. The Purkinje cells (arrow heads) are slightly or not labelled. The Bergmann glial cells (arrows) are not labelled. Scale bar: 25 μm.

MAP2b mRNA expression patterns. In the hippocampus, MAP2b mRNA expression was diffuse (Fig. 7B) indicating a somatodendritic localization while the MAP2d signal seemed concentrated and restricted only to the cell bodies of the granule neurones of the dentate gyrus (Fig. 7C) as indicated by cresyl violet staining (Fig. 7A). In order to confirm this differential distribution, we performed *in situ* hybridization experiments with digoxyginine-labelled MAP2b and MAP2d oligonucleotide probes on primary cultures of hippocampal neurones. As mentioned previously, cells were grown for 2 days in the presence of bFGF in order to increase MAP2d expression levels. All neurones showed extensive processes as revealed by differential interference contrast microscopy (Fig. 7D). In neurones with branched and two or more neurites, we identified the labelled neurites as dendrites using a MAP2 antibody (Fig. 7E). Very different subcellular distributions were observed when comparing MAP2b and MAP2d mRNA labelling. The MAP2b probe labelled both neuronal cell bodies and neurites (Fig. 7F,G,H). Occasionally, growth cones of neurites were also labelled (Fig. 7G). In contrast to the clear neuritic distribution of the MAP2b mRNAs, mainly the neuronal cell bodies (Fig. 7 J,K) and occasionally, very proximal areas of neurites (Fig. 7 L) were labelled following hybridization with the MAP2d probe. In these experiments, using double-labelling procedures (GFAP immunohistochemistry coupled to MAP2d *in situ* hybridization, not shown) we found no MAP2d labelling in the astrocytes that contaminate the cultures.

Discussion

We have previously cloned a novel small MAP2 variant MAP2d (Ferhat *et al.*, 1994a, b), and initiated a study on its potential contribution to the organization of microtubules and microfilaments (Ferhat *et al.*, 1996a). In the present report, we describe in rat brain the developmental, regional, cellular and subcellular distribution of the MAP2d mRNAs.

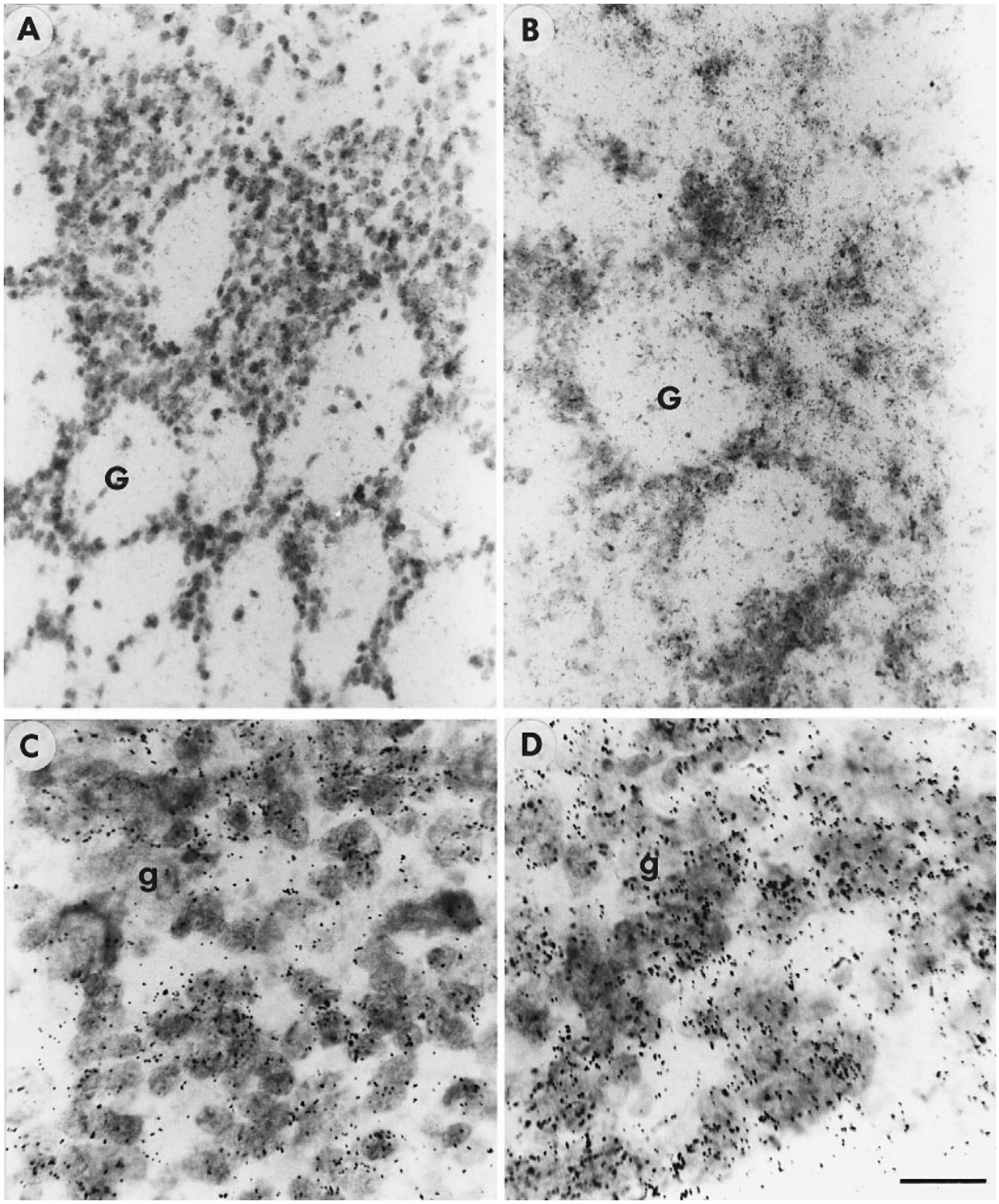

FIG. 5. Cellular localization of the MAP2d mRNAs during development of the olfactory bulb. Saggital sections of the olfactory bulb in P10 (A and C) and adult (B and D) rats. Lower levels of MAP2d mRNA are detected at P10 in the glomerular cells (G) and in the granule cells (g) than in adult. Scale bar: 25 µm.

MAP2d mRNA expression is delayed in the developing CNS and is predominantly expressed over MAP2c in the adult rat brain

Our RT-PCR analysis reveals that MAP2c and MAP2d isoforms are differentially regulated in rat brain during postnatal development. MAP2c mRNA levels are high at early postnatal stages and then decrease, a pattern that has been previously described in the developing brain (Charrière-Bertrand *et al.*, 1991; Ferhat *et al.*, 1994b; Kindler & Garner, 1994). In contrast, we show that MAP2d mRNA levels are low at birth and increase steadily during postnatal development to replace in part MAP2c in the adult rat brain, indicating that MAP2d is mostly involved in the late phases of maturation. In the adult brain, both MAP2c and MAP2d mRNA levels are low compared with the early phases of postnatal development. These observations differ slightly from those of Kindler & Garner (1994) following Northern blot analysis, showing that the amounts of the four repeat-6kb MAP2 mRNAs remain constant during early postnatal brain development. The developmental profile of the MAP2d protein is unclear at present. Doll *et al.*, 1993) reported that in adult rat brain the ratio of the 4-repeat MAP2c/3-repeat MAP2c mRNAs was not correlated with the ratio of the 4-repeat MAP2c/3-repeat MAP2c proteins. Indeed, immunoblot analysis reveals that these proteins are expressed in similar amounts in the adult rat brain (Doll *et al.*, 1993). However, other reports suggest that a small MAP2 protein, slightly larger in size than that expressed in the brain of the neonate, is the major variant in the adult rat brain (Leterrier & Eyer, 1992).

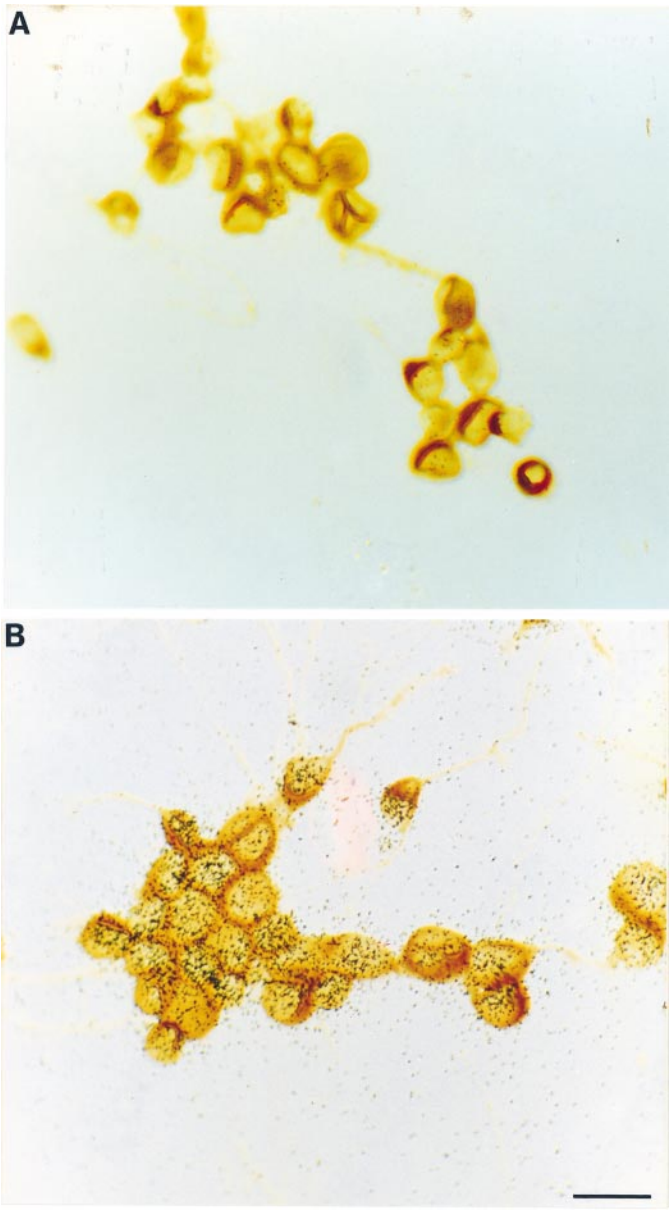

FIG. 6. Basic fibroblast growth factor (bFGF)-induced increase in MAP2d mRNA expression in cultured hippocampal neurones. The cells were cultured in the absence (A) or presence (B) of bFGF (25 ng/mL) for 2 days in serum-free medium. Cultures were hybridized with a ^{35}S-labelled MAP2d-specific oligonucleotide probe. Following *in situ* hybridization, the cells were counterstained with MAP2 antibodies and were clearly identified as neurones. Scale bar: 25 μm.

MAP2d is expressed in neurones during rat brain ontogeny

The strong homology between the MAP2d 31 amino acid insertion and the other microtubule binding domains of MAP2 and other MAPs (Ferhat *et al.*, 1994a) has hampered efforts to produce MAP2d-specific antibodies for a precise regional, cellular and subcellular localization of the protein. We have chosen to address the issue of MAP2d distribution using *in situ* hybridization with MAP2d specific oligonucleotide and cRNA probes. We show that our probes are specific for mRNAs encoding a four repeat MAP2: the probes we used, selected in the exon encoding the additional microtubule binding domain, do not recognize the related sequences encoding the three MAP2c microtubule binding domains. Other experimental data strongly suggest that the probes selected in the additional exon detect the mRNAs encoding a low-molecular weight (LMW) MAP2. Indeed, although Kindler & Garner (1994) have shown, using a northern blot approach, that in newborn rats the fourth repeat could be found in large (9.6 kb) MAP2 mRNAs, we (Ferhat *et al.*, 1994a; Ferhat *et al.*, 1994b) and others (Doll *et al.*, 1993; Forleo *et al.*, 1996) have shown that in the developing and adult rat brain, the exon encoding the additional microtubule repeat is barely detected if at all in high-molecular weight (HMW) MAP2 transcripts; to our knowledge, cDNAs encoding a four repeat HMW MAP2 have not been cloned in rat brain.

We have shown previously, using RT-PCR, that MAP2d mRNAs were expressed in structures of the CNS such as the cerebellum, the olfactory bulb, the hippocampus and cortex (Ferhat *et al.*, 1994b). This is confirmed in this report where *in situ* hybridization reveals strong MAP2d expression in the developing and adult cerebellum and olfactory bulb. At all developmental stages, labelling in the cortex was low compared with the latter two structures, and in the hippocampus, only the granule neurones of the dentate gyrus express MAP2d. During development, in all structures, the steady increase in the intensity of the hybridization signals suggest that MAP2d expression is associated with the maturation and terminal differentiation phases of the structures involved, rather than in their early development. However, MAP2d expression was also detected in some immature neurones such as the granule cells of the external granule layer of the cerebellum and in cells located in the molecular layer that could be migrating neurones.

In the adult brain, the MAP2d expression pattern is distinct from that of the MAP2b mRNAs whose distribution in the CNS is more homogeneous, and whose expression levels are high in the cortex and hippocampus, including in the pyramidal cell layer. Although one should be cautious in comparing the labelling obtained with two different probes, the MAP2d probe that we have used requires longer exposure times than the MAP2b probe. This observation was confirmed repeatedly as we have tried various MAP2d oligonucleotide probes as well as *in vitro* transcribed cRNA probes. These results indicate that the MAP2b and MAP2d mRNAs, the major MAP2 variants expressed in the adult rat brain, although transcribed from the same gene, differ substantially in their distribution and expression levels.

Our previous results (Ferhat *et al.*, 1994b) suggested that MAP2d expression was, in part at least, neuronal. Indeed, we have shown that MAP2d mRNA levels were substantially higher in primary neuronal cultures than in cultured glial cells. In addition, bFGF stimulation induced the expression of MAP2d in cultured hippocampal neurones (Khrestchatisky *et al.*, 1995) but not in cultured hippocampal astrocytes (L. Ferhat, unpublished results). The present study, using *in situ* hybridization, reinforces our previous assumptions and indicates that *in vivo*, MAP2d is mainly localized in well-characterized neuronal cells. Although low levels of MAP2d mRNAs were detected in the glial cultures (Ferhat *et al.*, 1994b), emulsioned brain slices reveal that *in vivo*, MAP2d mRNAs are not expressed in easily identifiable glial cells; the Bergmann glial cells of the cerebellum for instance are clearly not labelled.

Differential subcellular localization of the mRNAs encoding MAP2d and MAP2b

Besides the differences we observed in the levels of MAP2d and MAP2b mRNAs, and in the structures that expressed these mRNAs, it was also apparent from the respective *in situ* hybridization patterns that the subcellular distribution of the MAP2 mRNA variants was distinct. This was clearly the case in the hippocampus where MAP2d

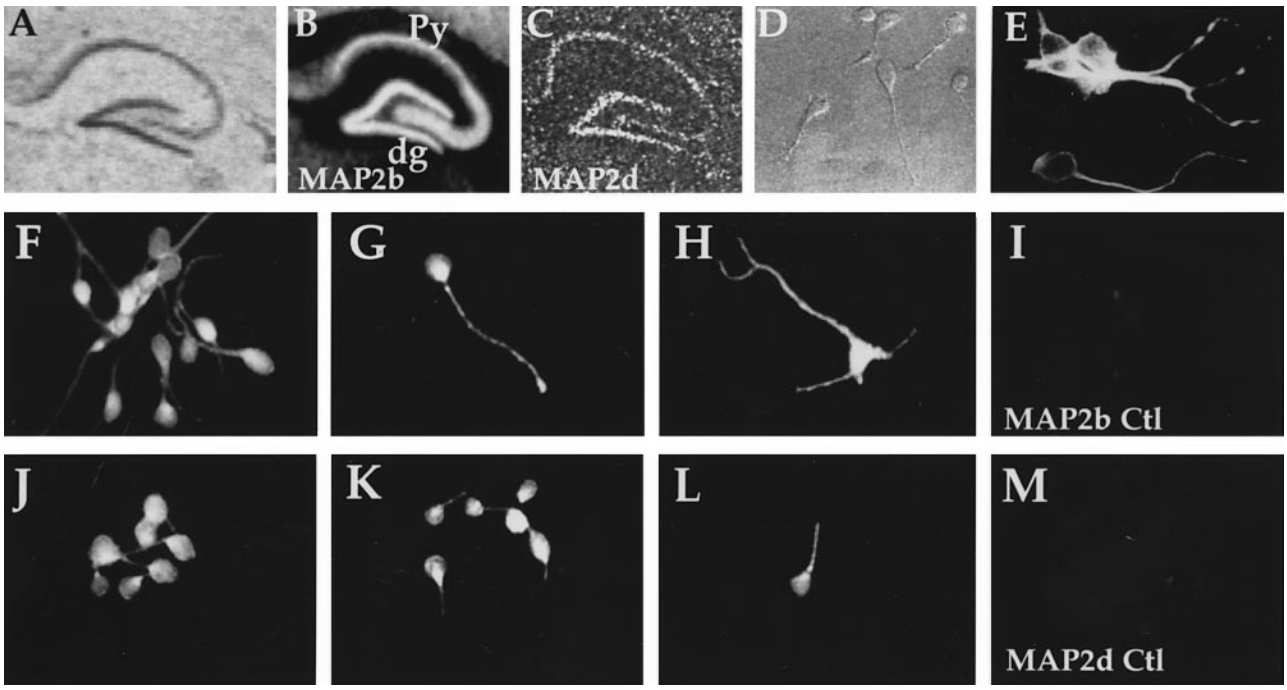

FIG. 7. Differential subcellular distribution of MAP2b and MAP2d mRNAs in the hippocampus and in cultured hippocampal neurones as revealed by *in situ* hybridization followed by digitized imaging of the brain sections, of the hybridization signal on the autoradiograms and of the cultured neurones. (A) Location of cell bodies of the hippocampal formation of an adult rat as shown by cresyl violet staining. (B) and (C) MAP2b and MAP2d mRNA distribution in the adult rat hippocampus: enlarged picture from digitized autoradiograms. The longer exposure times necessary for the MAP2d signal increase background when compared with MAP2b hybridization patterns. (D) Interferential-contrast image of hippocampal neurones after 2 days of bFGF (25 ng/mL) treatment. (E) Cultured hippocampal neurones stained with a MAP2 antibody reveal strong MAP2 labelling in branched neurites. (F–H) cultured hippocampal neurones hybridized with a digoxyginine labelled probe for MAP2b mRNAs. In (F), all neurones demonstrate MAP2b mRNA labelling in neurites. In (G), MAP2b mRNA labelling is observed in the growth cone of a neurone. In (H), MAP2b mRNA expression is observed in branched neurites of a neurone. (J–L) cultured hippocampal neurones hybridized with a digoxyginine labelled probe for MAP2d. In (J) and (K), in cultures identical to those shown in (D–H), none of the neurones demonstrate MAP2d mRNA labelling in neurites. In (L), occasionally, the proximal neurites of neurones are labelled with the MAP2d probe. (I, M) Absence of labelling obtained on cultured neurones (same density as in F and J) when a 200-fold excess non-labelled MAP2b and MAP2d oligonucleotides were added to the digoxyginine-labelled probes.

mRNAs appeared confined to the cell body layer of the dentate gyrus while the MAP2b mRNAs were distributed well beyond the cell body layers in a pattern characteristic of somatodendritic localization as previously described for MAP2 (Garner *et al.*, 1988; Kleiman *et al.*, 1990; Kleiman *et al.*, 1994). In order to confirm that differences in the subcellular distribution of the MAP2 variants were not related to their levels of expression, we further addressed this issue in cultured hippocampal neurones. MAP2d mRNA levels were substantially increased with bFGF, to levels that compare with those of MAP2b as judged by the intensity of the hybridization signals we obtained with both probes. We show clearly that the MAP2b mRNAs are expressed in growth cones and in neurites. In contrast, the MAP2d mRNAs are located exclusively in neuronal cell bodies, supporting the notion that MAP2d mRNA is not transported into dendrites. In agreement with our observations, Marsden *et al.* (1996) have demonstrated that in transgenic mice overexpressing MAP2c, the other small MAP2, the corresponding mRNA was localized only in cell bodies. The mechanisms that allow mRNAs to be transported to dendrites or confined to cell bodies are poorly understood. Papandrikopoulou *et al.* (1989) have suggested that the large MAP2 mRNA itself may contain the dendritic targeting signal within the 5′ and/or 3′ untranslated regions of the mRNA. Controlled cytoplasmic localization of mRNAs associated with the 3′-untranslated region has been reported for bicoid (bcd) transcripts in *Drosophila* embryos (Macdonald & Sruhl, 1988). Because the small MAP2 mRNAs appear absent from dendrites it is likely that they do not contain such dendritic targeting signals. However, to date, there is no sequence evidence to suggest that the 5′ and 3′ non-coding regions of the various MAP2 transcripts differ. Alternatively, the targeting signal may be included in the coding exon specific of the large MAP2. Thus, if it is proven that the MAP2d protein is localized in dendrites, it may likely depend on a targeting signal located within the protein itself as our results suggest that the MAP2d mRNA is not accumulated in dendrites.

Potential roles of MAP2c and MAP2d in the brain

The early expression and elevated levels of MAP2c and its partial replacement in the course of ontogeny by MAP2d raises the question of the relative roles played by both proteins in the developing brain. *In vitro* studies have shown that MAP2c is necessary for both cessation of cell division and neurite extension (Dinsmore & Solomon, 1991). The microtubule-assembling capacities of MAP2c and MAP2d have been evaluated, and as anticipated from similar studies with TAU variants containing three or four microtubule binding domains (Goedert & Jakes, 1990; Butner & Kirshner, 1991), MAP2d is more effective in promoting tubulin polymerization than MAP2c (Olesen, 1994). In addition, we have show in transfected cells that MAP2d is more effective than MAP2c in its ability to bundle and rearrange cellular microtubules and in its effectiveness in protecting microtubules and microfilaments from destabilizing agents (Ferhat *et al.*, 1996a). Thus, the delayed, but persistent expression of MAP2d in neurones in the process of maturation, supports the contention that

MAP2d is involved in the stabilization of the cytoskeleton in differentiating neurones.

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Abbreviations

bFGF	basic fibroblast growth factor
DTT	dithiothreitol
HMW	high molecular weight
MAP2	microtubule-associated protein-2
MEM	minimal essential medium
NR	nitrate reductase
PBS	phosphate-buffered saline
RT-PCR	reverse transcription polymerase chain reaction
SSC	standard saline citrate
SDS	sodium dodecyl sulphate
TdT	terminal deoxynucleotidyl transferase.

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