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Authors

Ferreira, A
Busciglio, J
Cáceres, A

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An immunocytochemical analysis of the ontogeny of the microtubule-associated proteins MAP-2 and Tau in the nervous system of the rat

Adriana Ferreira, Jorge Busciglio and Alfredo Cáceres

Instituto de Investigación Médica Mercedes y Martín Ferreyra, Córdoba (Argentina)

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The developmental distribution patterns of β -tubulin and the microtubule-associated proteins, MAP-2 and Tau, were studied by immunocytochemistry with monoclonal antibodies. The analysis of the in situ distribution of these proteins in embryonic brain tissue revealed intense immunoreactivity for β -tubulin in proliferative and migrating neuroblasts. On the contrary, no immunoreactivity for MAP-2 or Tau was detected in this neuroepithelium; specific immunostaining for these MAPs was only present in those neuroblasts which have reached their final destination within a developing brain area, and have initiated terminal differentiation, i.e. the sprouting of axons and dendrites. During the initial stages of neuritic outgrowth both MAPs were detected in the somatodendritic compartment of developing brain neurons; Tau was also present in axons. While the distribution of MAP-2 remained essentially the same throughout development, Tau was progressively lost from cell bodies and dendrites. This pattern of compartmentation was observed in pyramidal neurons of the cerebral cortex and hippocampus, as well as in cells of other brain regions (e.g. thalamus, hypothalamus, cerebral amygdala and tectum). It was not detected in cerebellar Purkinje cells which compartmentalize Tau to axons from the outset of neuritic differentiation, and in neurons of the Gasser ganglion which transiently express MAP-2 in axons. The expression and distribution of these MAPs was also analyzed in embryonic cerebellar and hippocampal pyramidal neurons grown in culture. Both MAPs were found in these cells as soon as 6 h after plating; they were also present in all of the neurites, axons and dendrites, that these cells extend after development in vitro for several days. With subsequence development (more than 4 days in vitro) MAP-2 was lost from axons, while Tau remained homogeneously distributed in both types of neurites. Taken collectively, the present results indicate that the development of the compartmentalized distribution of MAP-2 and Tau follows a complex pattern which is specific for each of these MAPs, and which varies as a function of the neuron type and the conditions under which the cell develops. In addition, the complex variations in the distribution of both MAPs during in situ and in vitro development make it unlikely that these proteins have a role in determining the fate of a neurite as an axon or a dendrite.

INTRODUCTION

During recent years it has become increasingly apparent that the synthesis of the microtubule-associated proteins (MAPs) is one of the critical events for driving the elaboration of axons and dendrites during neurogenesis^{17,25,37}. In favor of that view several biochemical studies have demonstrated an increase in microtubule-polymerization-promoting activity during brain development^{13,27,28,36}, a phenomenon which is associated with an increase in the complexity of the polypeptide species composition of the

MAPs^{13,23,27,28,36}. More important, both events follow a time course which is highly correlated with axonal and dendritic growth^{27,28,36}. A more direct demonstration of the involvement of the MAPs in neuritic growth has emerged from observations in neuronal tumor cell lines^{25,30}. These studies have shown that neuritic outgrowth is preceded and accompanied by a dramatic increase in Tau²⁵ and MAP-1 (refs. 25, 30) protein levels. The kinetics of these inductions correlate precisely with an increase in tubulin polymer mass and neuritic length; moreover, neuritic retraction is concurrent with a reduction in levels of MAPs

Correspondence: A. Cáceres, Instituto de Investigación Médica Mercedes y Martín Ferreyra, Casilla de Correo 389, 5000 Córdoba, Argentina.

and microtubule mass, but with only a slight decrease in total tubulin levels²⁵. This phenomenon suggests that the regulation of these associated proteins, but not tubulin, is the critical event for driving microtubule formation during neuritic outgrowth²⁵.

MAPs have also been implicated in other events related with neurogenesis. Thus, it has been proposed that different types of MAPs are specifically associated with the different phases of neuronal development^{27,28}. According to this proposal it is possible that the synthesis of a particular type of MAP might contribute to the overall differentiation of a multipotent stem cell into either a glial cell or a neuronal one; alternatively, they may contribute to determine the transition between a migrating neuroblast and a terminally differentiating one^{22,32}. Unfortunately, at present there is very little information in support of this notion since no immunocytochemical study has examined the cellular distribution of these proteins during early brain development.

It has also been proposed that the synthesis and subsequent compartmentation of a particular type of MAP at the beginning of neuritic formation may contribute to determine which neurite is going to become an axon or a dendrite^{9,17}. This idea is based on the fact that in mature neurons certain types of MAPs are preferentially localized in axons^{14,18,46} or dendrites^{10,17,19,20,24,31,37}, raising the possibility that the differences between these neurites arise in the asymmetric organization of neuronal microtubules. Unfortunately, the available evidence is controversial and limited to MAP-2 (refs. 9, 11, 16, 22, 23). For example, Bernhardt and Matus⁹ based on their studies of Purkinje cell development have made the interesting suggestion that the differential distribution of the high molecular weight MAPs, and specifically MAP-2 (ref. 11), determines which of the neuron's processes is going to become a dendrite or an axon. On the contrary, our own observations on the distribution of MAP-2 in embryonic hippocampal pyramidal cells grown in culture show quite the opposite²². At the outset of neuritic differentiation MAP-2 is present in all of the neuronal processes, axons and dendrites; it is only after several days in culture that MAP-2 disappears from the axons. While it is not yet known if axons of embryonic pyramidal neurons transiently express MAP-2 during *in situ* hippocampal development, our observations suggest that the

presence of this molecule does not preclude the fate of a neurite as an axon or a dendrite. However, it is clear that an analysis of the distribution of these proteins in the embryonic brain is highly desirable, since it is difficult to make comparisons between *in situ* and *in vitro* observations, particularly when using different model systems, and no immunocytochemical study has examined the localization of the MAPs at very early stages of brain development. This would undoubtedly contribute to a better understanding of microtubule compartmentation during neuronal development, and also might provide some clue about the role of these proteins in neuritic differentiation.

Thus, in the present study we have analyzed by immunocytochemistry the pattern of distribution of MAP-2 and Tau at different stages of neuronal development both *in situ* and *in vitro*. Besides providing an image of the intracellular distribution of these proteins during terminal neuronal differentiation^{12,33–35}, the analysis of the localization of the MAPs in embryonic brain tissue has allowed us to precisely determine the time of their expression during neurogenesis, and to establish if undifferentiated neuroblasts, either dividing or migrating, express any of these molecules.

MATERIALS AND METHODS

Preparation of tissue

Pregnant rats were used with the day of sperm-positivity designated as embryonic (E) day E 1; birth normally occurred on E 22. During the afternoon of E 15, E 17, E 19 and E 21, embryos were removed from pregnant females, decapitated, and either the entire head or the brain immersed in a fixative consisting of 4% paraformaldehyde–0.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2). Alternatively, the pregnant rats were anesthetized with ether, and the embryos perfused transcardially with the same fixative. The tissue was kept in fixative for 48 h, then washed in phosphate-buffered saline (PBS), and passed through a series of increasing concentrations of sucrose (12, 15 and 18%); it was finally kept in 30% sucrose at 4 °C until being processed for immunofluorescence. This tissue was sectioned in a cryostat at a nominal thickness of 20 µm and the sections mounted on slides previously coated with gelatin.

In addition, groups of 3 or more pups were also

sacrificed on the afternoon of postnatal (P) days P 1, P 6, P 10 and P 20. These rats were anesthetized by ether and transcardially perfused with a fixative consisting of 4% paraformaldehyde–0.25% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2). After fixation the brain was left in situ for 1 h, then removed from the skull and stored overnight in fresh fixative without glutaraldehyde; it was then processed in the same way as for the embryonic tissue, or was embedded in polyethylene glycol (PEG) according to the procedure of Smithson et al.⁴³ as previously described^{18,21}.

Preparation of cell cultures

Microexplants or dissociated cultures of cerebellar macroneurons were prepared according to the procedure described by Grau-Wagemans et al.²⁹ and Moonen et al.³⁹. Briefly, embryos were removed aseptically from pregnant rats on E 19 or E 21. They were transferred to dishes containing PBS where they were dissected under a stereomicroscope and freed of meninges. In some cases cerebella were obtained from newborn rats. For the microexplants the tissue was forced through a Nitex nylon mesh with a pore size of 215 μ m or cut into small fragments with iridec-tomy scissors. The microexplants were seeded onto poly-L-lysine-coated Aclar coverslips in 35 mm Falcon culture dishes at a density of one cerebellum per milliliter of culture medium. The tissue culture medium employed was Eagle's Minimum Essential Medium supplemented with the N 2 mixture of Bottestein and Sato¹⁵, plus 0.1% ovalbumin^{4–7}. For the dissociated cell cultures, the cerebellar fragments were incubated with 0.25% trypsin solution, then rinsed with culture medium, and finally passed through a 40 μ m pore size Nitex mesh. The resulting cell suspension was also plated onto poly-L-lysine-coated Aclar coverslips at a density of 1,000,000 cells/35 mm culture dish. They were maintained with a culture medium identical to the one previously described plus cerebellar microexplant conditioned medium²⁹. The conditioned medium was prepared and diluted to allow dissociated cerebellar macroneurons achieve maximal survival as described by Grau-Wagemans et al.²⁹. Finally, dissociated hippocampal cell cultures were prepared as previously described^{4,5,22}. All types of cultures were prepared for immunocytochemistry using fixation schedules identical to those previously

described^{20,22}.

Production and characterization of monoclonal antibodies

A detailed description of the preparation and characterization of the monoclonal antibodies against MAP-2 (clone AP9 and AP14) and Tau (Tau-1) used in this study has been presented previously^{13,14,18,19,40,41}. The binding specificity of each clone was examined by solid phase enzyme-linked immunoassay, and cross-reactivity to other proteins was tested by reacting the antibodies with electroblots of rat hippocampus or cerebellum. MAP-2 monoclonal antibodies (clone AP9 and AP14) recognized both isoforms of the protein (MAP-2a and b)^{13,19,40,41} while Tau monoclonal antibody (clone Tau-1) recognized all of the Tau species (Tau a, b, c, d and e)^{14,18}.

Immunocytochemical procedures

Frozen sections of embryonic or postnatal brain tissues were processed for immunofluorescence according to the procedure described by De Camilli et al.²⁴. The sections were preincubated in normal goat serum (diluted 1:6) or 5% bovine serum albumin for 30 min, and then incubated in primary antibodies for 3 h at 37 °C. The following primary antibodies were used in this study: Tu9B, Tu12, and Tu27 (β -tubulin monoclonal antibodies); AP9 and AP14 (MAP-2 monoclonal antibodies); and Tau-1 (Tau monoclonal antibody). After incubation in primary antibody, the sections were washed in PBS, and then incubated in goat anti-mouse IgG rhodamine conjugate for 2 h at 37 °C. Slides were finally covered with 95% (v/v) glycerol and viewed with epi-illumination fluorescence optics. PEG-embedded tissue was sectioned in a rotatory microtome at a nominal thickness of 40 μ m. Free-floating sections were processed for immunocytochemistry using the immunoperoxidase method of Sternberger⁴⁴ as described^{18,21}. Sections treated with normal serum rather than primary antibody were used as controls. Immunocytochemistry of cell cultures was performed as previously described^{20,22}.

RESULTS

The principal observations of this study involve neurons in the hippocampal formation and cerebel-

lum. These areas have been chosen for analysis because of their simple cyto- and dendro-architectonics, as well as for their well-known anatomical and histological developmental pattern^{2,3,8,33}. In addition, several recent immunocytochemical studies have established the pattern of distribution of MAP-2 and Tau in mature neurons of these areas^{10,14,}

17–20,24,34,37,46, thus allowing comparisons to be made between the adult pattern and the developmental one. Also, direct comparisons between in situ and in vitro observations are possible, since hippocampal and cerebellar neurons can differentiate and elaborate axons and dendrites in vitro^{4–7,29,39}. Summarizing, several aspects make the hippocampus and cerebel-

Fig. 1. Distribution of β -tubulin (A, B), MAP-2 (C) and Tau (D) in the embryonic brain (E 15). MAP-2- and Tau-induced immunofluorescence are only observed in terminally differentiating neurons (tdn), while tubulin is homogeneously distributed in the neuroepithelium (ne) as well as in tdn. VL, ventricular lumen; directional arrows give orientation (coronal sections). $\times 97.5$ (A, C, D); $\times 195$ (B).

Fig. 2. Distribution of β -tubulin (A, B), MAP-2 (C, D) and Tau (E, F) in the hippocampal primordium (hp) at E 15. Note the absence of MAP-2- and Tau-induced immunofluorescence in the neuroepithelium (ne) of the hp. LV, lateral ventricle; np, neocortical primordium; cp, choroid plexus. $\times 91$ (A, C, E); $\times 182$ (B, D, F).

lum particularly suitable for studying the principles of MAP-2 and Tau distribution during ontogenesis. However, in order to obtain a more detailed image of the distributional pattern(s) of these proteins in the developing nervous system, other major gray and white matter areas (e.g. cerebral cortex, thalamus, peripheral ganglion, etc.) have also been analyzed.

Distribution of tubulin, MAP-2 and Tau in the developing nervous system (in situ observations)

Prior to describing the localization of the MAPs within a particular area of the developing brain, the distributional pattern of these molecules on E 15 (the youngest gestational day analyzed) will be provided. In these embryos, oval or spherical cells located in the proliferative periventricular zone were easily identified with anti-tubulin staining (Fig. 1A, B). The cells within this region were intensely immunofluorescent for β -tubulin in all of the brain areas analyzed. Also, bright immunofluorescence was observed in migratory cells adjacent to the proliferative zone, as well as in those cells which have reached their final position within a particular area (Fig. 1A). On the other hand, a quite different pattern was detected with the monoclonal antibodies against MAP-2 or Tau. In fact, no immunofluorescence for any of these proteins was observed in the proliferative cells of the periventricular wall or in the migrating ones (Fig. 1C, D). However, intense immunofluorescence for both MAPs was observed in cells which have reached their final destination in the brain (Fig. 1C, D). This resulted in a sharp demarcation between the neuroepithelium and the cells which have initiated the process of terminal neuronal differentiation (Fig. 1).

Hippocampal formation. On E 15 the neuroepithelium of the dorsomedial wall of the telencephalon curves into the lateral ventricle to form the hippocampal primordium⁸. The cells located within that region were intensely immunofluorescent for β -tubulin (Fig. 2A, B), while no immunostaining was detected for MAP-2 (Fig. 2C, D) or Tau (Fig. 2E, F). A similar pattern was observed on E 17, with the only exception that a few scattered cells, which have reached their final position in the hippocampal primordium, were lightly immunofluorescent for both MAPs (data not shown).

By E 19 the hippocampal formation has developed

considerably, and the characteristic shape of the adult hippocampus is already evident. Many of the cells have reached their final position and initiated the process of axonal and dendritic formation; in addition, several major axonal systems (e.g. fimbria, parallel fibers, etc.) are quite evident at this embryonic day. All of these elements, as well as the neuroepithelium were intensely immunofluorescent for β -tubulin (Fig. 3). On the other hand, the pattern of

Fig. 3. A: thionin-stained section of the developing hippocampus at E 19; note the intense staining of cell nuclei in the neuroepithelium (ne) and pyramidal cell layer (pcl). B: adjacent section immunoreacted with a monoclonal antibody against β -tubulin. Tubulin immunoreactivity is localized in the neuroepithelium (ne), axonal fibers (ax), and in pyramidal cells (pcl); the fimbria (Fi) is also heavily stained. $\times 62.5$.

Fig. 4. Distribution of MAP-2 in the developing hippocampus at E 19 (A–C). Intense immunoreactivity for MAP-2 is only detected in developing pyramidal cells (pc). Note the absence of staining in the neuroepithelium (ne), and in the fimbria (Fi). ×100 (A); ×200 (B, C).

Fig. 5. Distribution of Tau in the developing hippocampus at E 19 (A-C). Tau immunoreactivity is localized in developing pyramidal cells (pc), axonal fibers entering the hippocampus (Ax), and in the fimbria (Fi); note the absence of staining in the neuroepithelium (ne). $\times 89$ (A); $\times 178$ (B, C).

distribution of MAP-2 was strikingly different. Intense immunofluorescence for this protein was detected in the cell bodies and growing dendrites of pyramidal cells (Fig. 4), while no immunostaining was observed in axonal fibers (Fig. 4A, B), or in the neuroepithelium (Fig. 4A, B). Within the dendrites the immunostaining was homogeneous along the whole extent of the growing processes (Fig. 4C). In the case of Tau, intense immunofluorescence was detected in axonal fibers entering the hippocampus (Fig. 5); in addition, specific immunofluorescence was observed in neuronal cell bodies and growing dendrites of pyramidal cells, while no immunostaining was detected in the neuroepithelium (Fig. 5).

The pattern of distribution of these MAPs during the first postnatal week was very similar to that previously described for the embryonic hippocampus. The major differences were the slight decrease of MAP-2 immunostaining in the cell bodies of pyramidal neurons and the disappearance of Tau immunoreactivity from the growing dendrites. By day P 10 the pattern of distribution of MAP-2 and Tau was identical to that observed in the adult hippocampus^{18,19}. MAP-2 immunofluorescence was primarily found in dendrites, while a moderate degree of immunostaining was detected in the cytoplasm of neuronal perikarya (Fig. 6A); on the contrary, Tau immunoreactivity was exclusively localized to axonal fibers, and no immunostaining was observed in cell bodies or dendritic processes (Fig. 6B; see also refs. 14 and 18).

Cerebral cortex. The fluorescent pattern of the embryonic cerebral cortex immunostained for MAP-2 or Tau is illustrated in Fig. 7. Again, in this brain region, specific fluorescence could only be detected in cells which have reached their final position and initiated the process of terminal differentiation. Thus, by E 17 a single layer of MAP-2- or Tau-immunofluorescent cells was detected in the cortical primordium, while no immunostaining was present in the neuroepithelium (Fig. 7B, C); the incipient growing dendrites of these cells were also immunoreactive for both types of MAPs (e.g. see Fig. 7B). Dendritic labeling was clearly evident by E 19, where MAP-2 or Tau immunoreactivity was detected along the whole extent of the growing apical dendrites of cortical pyramidal neurons (Fig. 8A–C); fluorescence intensity for both MAPs, as well as for β -tubulin, was remark-

Fig. 6. Distribution of MAP-2 (A) and Tau (B) in the developing hippocampus at P 20. Intense immunoreactivity for MAP-2 is detected in apical dendrites of CA₃ pyramidal cells (pcd). On the contrary, Tau immunoreactivity is localized in mossy fibers (mf) and parallel fibers; note the absence of immunostaining in pyramidal cells (pcl). $\times 188$.

ably constant along these growing dendrites (Fig. 8). A progressive distinction between the pattern of distribution of MAP-2 and Tau became evident during the first postnatal week; it basically involved changes in the distribution of Tau immunoreactivity. Thus, a considerable decrease in Tau immunofluorescence was observed in cortical cell dendrites and neuronal perikarya; in addition intense immunofluorescence was observed in axonal fibers entering the cerebral

Fig. 7. Distribution of β -tubulin (A), MAP-2 (B) and Tau (C) in the developing cerebral cortex at E 17. MAP-2- and Tau-induced immunofluorescence is only observed in terminally differentiating neurons (tdn) and the incipient growing neurites (B). CNE, cortical neuroepithelium; VL, ventricular lumen; ne, neuroepithelium. $\times 304$ (A); $\times 190$ (B, C).

Fig. 8. Distribution of β -tubulin (A), MAP-2 (B) and Tau (C) in the developing cerebral cortex at E 19. Intense immunostaining is observed in cortical pyramidal cells (C-pc) and their growing dendrites (pcd). $\times 292$.

cortex (Fig. 9A). By the end of the first postnatal week the pattern of distribution of the two MAPs was identical to that observed in the adult hippocampus; Tau was exclusively found in axons and MAP-2 in dendrites. In this regard, it is noteworthy that at any time of cortical development we detected MAP-2 immunoreactivity in axonal fibers.

Other brain regions. The pattern of distribution of MAP-2 and Tau immunoreactivity described thus far was found to be similar in a large number of other brain regions analyzed (e.g. the thalamus, hypothalamus, cerebral amygdala, tectum, etc.). Thus, with a few exceptions (e.g. the cerebellum and peripheral ganglion, see following sections), both MAPs were initially detected in the somatodendritic compartment of the immature neuron; while MAP-2 remained in this compartment, Tau only localized temporarily on it. It is also important to emphasize that no MAP-2-immunoreactive axons were observed at

any time of development in any of these brain areas, despite the fact that several axonal tracts were immunoreactive for β -tubulin or Tau as early as E 15.

Cerebellum. No evidence of MAP-2- or Tau-induced immunofluorescence was observed in the proliferative neuroepithelium or external granule cell layer of the embryonic cerebellum (Fig. 10A); however, moderate immunofluorescence for Tau was detected in axonal fibers entering the central portion of the internal granule cell layer. A quite similar pattern was observed on day P 1, with the only exception that several patches of Tau- (Fig. 10B) or MAP-2-immunoreactive cells were present in the internal granule cell layer. By day P 4, both MAPs as well as tubulin were observed in Purkinje cell bodies; striking differences in the pattern of distribution of the proteins were detected at the end of the first postnatal week. By that time, immunofluorescence for Tau was localized in axonal fibers of the molecular layer, while

Fig. 9. Distribution of Tau (A) and MAP-2 (B) in the developing cerebral cortex at P 1. Note the decrease in Tau-induced immunofluorescence in pyramidal cell dendrites (pcd), and the intense labeling of axonal tracts (Axt) entering the cerebral cortex. MAP-2 displays a quite different pattern: note the strong labeling of growing dendrites (pcd), pyramidal cells (C-pc), and the absence of staining in Axt. $\times 276$.

dendrites remained unstained (Fig. 10C); on the contrary, intense immunoreactivity for MAP-2 was present in the apical cone and distal dendrites of developing Purkinje cells (Fig. 11). In the case of tubulin, immunoreactivity was observed in parallel fibers as well as in the cell body and primary dendrites of Purkinje cells, while secondary and tertiary dendrites remained unstained. The same type of differences were present at the end of the third postnatal week; by that time the distribution pattern of the 3 molecules was identical to that previously described by several authors for the adult cerebellum^{10,14,19} (see also Fig. 11).

Peripheral ganglion. For analyzing the distribution of the MAPs in ganglionic nerve tissue we have fo-

cused our attention on the Gasser ganglion. In the 15-day-old rat embryo (Fig. 12) the large trigeminal ganglion is easily recognized, thus providing an excellent opportunity to analyze the pattern of distribution and compartmentation of MAP-2 and Tau. Intense immunofluorescence for both MAPs as well as for tubulin was detected in the cell bodies of the ganglionic neurons at E 15 (Fig. 12A, C, E). The 3 proteins were also present in axonal fibers entering or leaving the ganglion (Fig. 12A, C, E); the relative intense fluorescence of the proteins in these fibers was almost identical. Significant differences in the distribution pattern of both types of MAPs were detected two days later (E 17). By that time Tau had almost disappeared from the cell bodies (Fig. 12D), while in-

Fig. 10. Localization of Tau (A–C) and tubulin (D) in the developing cerebellum. A: note the absence of Tau-induced immunofluorescence in the external (egcl) and internal (igcl) granule cell layers of the embryonic cerebellum (E 19). B: distribution of Tau immunoreactivity at P 1; note the labeling of cells in the igcl, and in axonal tracts (Axt). C: distribution of Tau immunoreactivity at P 6; note the labeling of the molecular layer, and the absence of staining in Purkinje cell bodies (Pc) and their dendrites (Pcd). D: distribution of β -tubulin at P 1. $\times 190$.

tense immunofluorescence was present in axonal fibers (Fig. 12D). On the other hand, a dramatic decrease of MAP-2 immunostaining was observed in

axonal fibers; this was particularly evident in the axonal initial segments (Fig. 12F), while the distal portions of the axons remained slightly immunofluores-

Fig. 11. Distribution of MAP-2 (A) and Tau (B, C) in the developing cerebellum. A: detail of MAP-2 distribution in the cerebellar cortex (P 10); note the differential labeling of Purkinje cell dendrites (Pcd). B, C: detail of Tau distribution in the cerebellar cortex (P 20). Note the absence of staining in Purkinje cell dendrites (Pcd), and the strong labeling of the axonal initial segments of these cells (Pca, arrows); Tau immunoreactivity is also observed in parallel fibers coursing through the molecular layer (ML). $\times 320$.

Fig. 12. Distribution of β -tubulin (A, B), Tau (C, D) and MAP-2 (E-G) in the developing Gasser ganglion (Gg). Intense immunostaining is observed in ganglionic neurons (gn) and in axonal tracts (Axt) at E 15 (A, C, E). Note the decrease in Tau immunoreactivity in the cell bodies of gn and the intense immunofluorescence of Axt at E 17 (D). Also note the disappearance of MAP-2 immunoreactivity from the gn and the axonal initial segments (F); light immunoreactivity is observed in the distal portions of the axons (G). Axb, axonal bundles. $\times 175$ (A, C, E-G); $\times 87.5$ (B, D).

cent (Fig. 12G). In addition, no MAP-2 immunoreactivity was detected in the cell body of the ganglionic neurons (Fig. 12F). By E 19 MAP-2 has completely

disappeared from axons and Tau from the cell body; tubulin-induced immunofluorescence was identical as on E 15 (Fig. 12).

Fig. 13. Distribution of Tau in cerebellar neurons after development for 1 h (A, B) or 6 h (C, D) in culture. A: bright field micrograph of a microexplant prepared from a 19-day-old rat embryo showing the absence of Tau immunoreactivity; the insert shows the phase contrast image of the cells in the microexplant. B: bright field micrograph of a microexplant prepared from a 1-day-old rat showing intense Tau immunoreactivity. C, D: bright field micrographs of microexplants prepared from the cerebellum of a 19-day-old rat embryo. Tau immunoreactivity is observed in neuronal cell bodies (C) and in the incipient growing neurites (D). $\times 197.5$.

Distribution of Tubulin, MAP-2 and Tau in the developing nervous system (in vitro observations)

In agreement with previous reports²⁹, cerebellar macroneurons survive and differentiate when cul-

tured as microexplants in serum-free medium (SFM) or as dissociated cells in SFM plus cerebral cortex conditioned medium. When cultured as microexplants, the cells extend numerous long radially

Fig. 14. Distribution of MAP-2 (A, B) and Tau (C, D) in cerebellar microexplants after development in culture for 24 h. The phase contrast micrographs (A, C) show the cells in the microexplant (me) extending numerous long radially oriented processes; the bright field micrographs illustrating the distribution of MAP-2 (B) and Tau (D) immunoreactivity, show intense staining of cell bodies and the processes which emerge from the microexplant. $\times 312$.

Fig. 15. A: dissociated cerebellar neurons after development in culture for 24 h, stained with an antibody against MAP-2 (AP-14); note the labeling of the incipient dendrites (open and small arrows), and of axonal processes (solid arrow). B, C: cerebellar neuron after 4 days in culture. The phase contrast micrograph illustrates the processes emerging from this neuron; MAP-2 staining, seen in the bright field micrograph (C) is only present in the dendrite (open arrow), while the axon remained unstained (solid arrow). D: cerebellar macroneuron after 4 days in culture stained with an antibody against Tau (Tau-1); note the labeling of the dendrites (den; open arrow) and of the axon (solid arrow). E: distribution of Tau immunoreactivity in cerebellar microexplants after 4 days in culture; note the strong labeling of the cells in the microexplant (me) and of the axonal bundles (ab). $\times 195$ (A–C); $\times 468$ (D); $\times 97.5$ (E).

oriented neurites as soon as 24 h after plating (Figs. 13 and 14). This aspect remains essentially the same for at least 10 days; in the present study no attempt was made to achieve longer term cultures.

A similar phenomenon occurs when the cells are allowed to survive and differentiate as dissociated cells in SFM plus conditioned medium. Under these conditions a detailed examination of the morphological development of these neurons is possible. As in the case of the microexplants, the dissociated cells attach to the substratum and initiate the process of neurite outgrowth shortly after plating. Within the first 48 h the cells elaborate two distinct classes of neuronal processes; in addition, two different populations of nerve cells are easily identifiable by that time. The most common cell types are oval or spherical neurons with a mean diameter of approximately 10 μm , and one or two processes which at this stage are 20–50 μm in length. The other type of neuron has a more elaborate shape; these neurons are at least 20 μm in diameter, and they typically extend two well-differentiated types of neuronal processes. One neurite type has the general characteristics of axonal processes, i.e. they extend a great distance from the cell body and do not taper. The other neurites which are thicker, taper with distance, branch at Y angles and acquire an elaborate branching pattern (e.g. see Fig. 15D).

In order to determine if MAP-2 or Tau are expressed under in vitro conditions by embryonic cerebellar neurons, we examined cultures 1 or 6 h after plating. As is shown in Fig. 13A, no MAP-2 or Tau immunoreactivity was detected in these cells 1 h after plating while intense specific staining was observed 5 h later (Fig. 13C, D). A similar experiment conducted in cultures prepared from newborn rat cerebellum revealed that nearly all of the cells reacted with the antibodies against MAP-2 or Tau, as soon as 1 h after plating (Fig. 13B). The pattern just described was observed independent of the type of culture employed (e.g. microexplants or dissociated cells).

As previously mentioned, within a day after plating most neurons had begun to extend neurites. Immunostaining of these cultures with antibodies against β -tubulin, MAP-2 or Tau revealed intense reaction product in the perikarya and processes, both axons and dendrites, of these cells. Examples of mi-

croexplants or dissociated cells after 1 or 2 days in vitro are shown in Figs. 14 and 15A. Light immunostaining for all these proteins was observed in flattened regions of the cytoplasm as well as in growth cones. A similar pattern was detected in cells which were allowed to survive 3 days in culture, while after that time a distinction between the distribution of Tau and MAP-2 became evident (Fig. 15B–E). Thus, Tau immunoreactivity remained uniformly distributed in axons and dendrites at all the stages of development we analyzed, while MAP-2 became restricted to the somatodendritic compartment of the neuron. A similar pattern of development was observed in hippocampal pyramidal cell cultures (data not shown).

DISCUSSION

MAP-2 and Tau as markers of terminal neuronal differentiation

The present study provides a detailed analysis of the distribution of β -tubulin, MAP-2 and Tau, in the developing nervous system of the rat. The analysis of the in situ distribution of both types of MAP in the embryonic brain provides direct evidence for the notion that these proteins are only expressed by differentiated neurons. In fact, our results show that MAP-2 and Tau are expressed by *prospective* neurons which have reached their final destination within a particular brain region, and have initiated the process of terminal neuronal differentiation^{12,33–35}. Thus, MAP-2 and Tau seem to be similar to other neuron-specific molecules which also appear in the nervous system after cessation of DNA synthesis, and withdrawal from mitosis and migration^{34,38,42,45}.

In order to analyze the biological significance of such a pattern several possibilities have to be considered under the premise that lack of immunostaining does not preclude absence of antigen (see refs. 19 and 21). For example, it may be that MAP-2 or Tau are expressed by either dividing or migrating neuroblasts in such a low amount that our immunohistochemical procedure cannot detect them; the masking of the AP9, AP14, or Tau-1 epitopes by protein–protein interactions in these cells may also account for the observed pattern. Alternatively, it may be that both MAPs have a labile association with tubulin in the immature neuroblasts, becoming extractable un-

der the conditions used to prepare the tissue for immunocytochemistry. While it is difficult to exclude any of these possibilities for explaining the cell type-specific distribution of the MAPs during early brain development, several aspects of our observations favor the view that these proteins are in fact only expressed by differentiated neuroblasts, and not by proliferative or migrating ones. Thus, this localization pattern is consistently detected in any developing area, either of the embryonic or early postnatal brain, independent of the type of fixation, washing schedule, or antibody concentration, clearly indicating that it is not the consequence of some artifact related to the immunohistochemical procedure. Besides, the transition between MAP-2 or Tau non-immunoreactive neuroblasts to immunoreactive ones is sharp rather than progressive suggesting that a major change in the expression of these proteins takes place when the neuroblasts reach their final destination. This characteristic, plus the fact that the appearance of the MAPs is concurrent with an increase in β -tubulin immunoreactivity (see also ref. 26), and the initiation of axonal and dendritic outgrowth are of particular relevance in view of previous biochemical and immunocytochemical studies in neuronal tumor cell lines which have shown that these MAPs are only expressed in significant amounts by cells which have initiated neuritic formation^{17,25,30,32}. Then, on the basis of the present evidence, it is likely that the expression of MAP-2 and Tau by in situ developing neuroblasts is one of the earliest and perhaps key events for switching on the initial phases of terminal neuronal differentiation, i.e. the sprouting of axons and dendrites. On the other hand, it is unlikely that these proteins have a role in determining the commitment of a cell to the neuronal lineage since they seem to be almost absent in proliferative and migrating neuroblasts. However, it is worthy of note that the absence of MAP-2 or Tau immunoreactivity in immature neuroblasts may not imply that the cells lack the information and/or machinery to express these proteins. Thus, in situ embryonic cerebellar neuroblasts do not display MAP-2 or Tau immunoreactivity at E 19; however, if cerebellar cells of this gestational day are grown in culture intense specific immunostaining for both MAPs is detected within 6 h after plating, a phenomenon that is accompanied by neurite formation. This pattern is quite different from the one ob-

served in situ where significant expression of these MAPs by cerebellar cells is only observed after birth. These results suggest that the capacity to express these proteins is dependent upon the conditions under which the cells develop, rather than on a pre-established endogenous program. It will now be of considerable interest to begin exploring which are the factors that regulate the expression of these proteins during early neuronal development both in situ and in vitro.

The distribution of MAP-2 and Tau in developing neurons

Our results indicate that the development of the compartmentalized distribution of MAP-2 and Tau, which is characteristic of mature neurons^{10,14,17,19,20,22,23,31,37,46}, follows a complex pattern which is specific for each of these MAPs, and which varies as a function of the type of neuron and the conditions under which the cell develops.

Of the MAPs analyzed, MAP-2 is the one which has the simplest pattern, being restricted to the somatodendritic compartment of in situ developing neurons from the outset of neuritic differentiation and growth (see also ref. 9, 16 and 17). This is important since it rules out the possibility of a transient localization of MAP-2 in axons during normal development in situ, as it occurs in the cells of the Gasser ganglion (peripheral neurons), or in central neurons which develop in vitro^{1,23} (this study).

On the other hand, Tau has a more complex pattern, having either a restricted axonal distribution, or a widespread one, localized both in axons and dendrites; in this case, it is not until the dendrites have developed considerably that the protein begins to disappear from this compartment. Our observations also indicate that the dendritic distribution of Tau is cell type specific; thus, in developing cortical pyramidal neurons Tau colocalizes with MAP-2 and tubulin along the whole extent of the growing dendrite, while in cerebellar Purkinje cells the protein is detected in axons and not in dendrites. The transient dendritic localization of Tau in pyramidal neurons as opposed to its absence in Purkinje cells is probably reflecting other important differences in the organization of microtubules between these two types of neurons. In this regard, it is worth noting that the dendrites of Purkinje cells seem to develop with few if any micro-

tubules^{2,3}, while these structures are prominent elements in the developing dendrites of pyramidal neurons²². Besides, our results indicate that the pattern of intradendritic distribution of MAP-2 is also different in these two types of nerve cells.

While at present it is difficult to precisely determine the biological significance of the differential distribution and organization of microtubular proteins between Purkinje and pyramidal neurons, it is likely that they are directly linked with the different morphologies and growth characteristics of their dendrites. Moreover, it is also possible that they reflect or are the consequence of the different environment under which these cells develop. An obvious prediction of such an hypothesis is that the pattern of distribution and organization of microtubular proteins would change as a function of the environment under which the cell develops.

The present study provides evidence which favors this view. Thus, hippocampal pyramidal neurons and cerebellar macroneurons when developing in culture display a distribution pattern of MAP-2 and Tau which is quite different from the one expressed by their counterparts in situ. Similar modifications in the distribution pattern of MAP-2 have been reported for cerebellar microneurons (granule cells) grown in culture^{1,17,23}.

Two basic possibilities come into mind regarding a biological origin of these modified patterns. For example, it may be that they are part of an adaptative response of the 'transplanted' neurons to the new environment created by the in vitro conditions; alternatively, it is possible that the reported modifications are the consequence of greatly disrupting the normal cellular interactions and/or other type(s) of environmental signals which operate in situ. The possibility of an environmental regulation of the distribution patterns of these molecules during axonal and dendritic growth, does not conflict with and/or exclude the existence of an endogenous control^{7,8,22}; it rather complements it. Moreover, it is likely that there is a balance between endogenous and exogenous regulation of distribution of MAPs, which also is a function of the type of neuron, as well as of the type of MAP. For example, in the case of pyramidal neurons, the distribution pattern of MAP-2 is modified by growing the cells in vitro; however, and regardless of the precise mechanism, the cells are able to compart-

mentalize MAP-2 to dendrites even when grown in isolation, clearly indicating that at least this aspect of the organization of microtubules can be controlled by an endogenous mechanism²². A similar phenomenon seems to occur in cerebellar macroneurons grown in culture. In contrast, the compartmentation of MAP-2 in cerebellar granule cells may be determined by cell interactions^{1,17,23}. When granule cells are grown in the absence of cellular contacts they express MAP-2 in axons-like processes¹; on the other hand, when they are grown at higher densities, which allow extensive cell aggregation, the protein becomes restricted to the cell body and does not enter the axonal processes^{1,17,23}. The situation seems to be more complex for the regulation of the compartmentation of Tau; our results clearly show that it does not depend on an endogenous mechanism, or on the cell-cell interactions that take place in the cultures we have used in this study. Perhaps, the compartmentation of this molecule is highly dependent on the existence of specific cell interactions which do not operate or are absent in the present in vitro conditions. Regardless of the precise reason, it is clear from our observations that the distribution of MAP-2 and Tau are *differentially regulated*, not only within a given neuronal population, but also between different neuron types.

The final issue to consider is related with the role of these MAPs during neuronal development. Unfortunately, the complex variations in the distribution pattern of MAP-2 and Tau during development make it extremely difficult to propose a role for these proteins in neuritic differentiation and growth. It has been proposed that the compartmentation of these MAPs at the beginning of neuritic formation may contribute to determine which neurite is going to become an axon or a dendrite^{9,11,17}. The present evidence does not favor this view. For example, the distribution pattern of MAP-2 and Tau in cells grown in culture clearly shows that the presence of these proteins within a particular neuronal domain at the beginning of differentiation does not modify what seems to be the 'pre-established' fate of the neurite. Thus, axons can contain MAP-2 and still differentiate as such; conversely, dendrites may contain Tau and still maintain the characteristics of these processes. While these conclusions are mainly based on the observation of the development of the cells in culture, some of our in situ observations fully support

this notion. In this regard, the transient expression of MAP-2 by in situ developing neurons of the Gasser ganglion is particularly relevant; in fact, our results show that these cells, which lack dendrites, are able to express MAP-2 and send it to the growing neurites without modifying their axonal fate. In addition, the observations in the Gasser ganglion clearly indicate that the expression of MAP-2 is insufficient to make a cell produce neurites which differentiate as dendrites. It seems clear that the role these MAPs have in development is not primarily related with the differentiation of a neuron's processes. This does not mean that other components of microtubules may have such a role, or that a specific pattern of microtubular proteins within a given neurite, specifies its fate

as an axon or dendrite. A major goal of future studies will be to determine which is the exact role of MAP-2 and Tau during neuronal development.

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