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Expression of microtubule-associated proteins MAP2 and tau in cultured rat brain oligodendrocytes

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Abstract. Oligodendrocytes in culture are characterized by large membranous sheets containing an elaborate network of microtubules. Microtubule-associated proteins (MAPs) participate in microtubule stability and the regulation of the cellular architecture. We have investigated the expression of two major groups of MAPs, MAP2 and tau, in cultured rat brain oligodendrocytes. Alternatively spliced isoforms of mRNAs encoding MAP2 and tau were assessed by means of reverse transcription and polymerase chain reaction using a newly designed set of MAP2- and tau-specific primers. The data were compared with data obtained with cultures of rat brain astrocytes and rat cerebral neurons, and adult rat brain. The results show that oligodendrocytes, similarly to neurons, express mainly MAP2c transcripts containing three microtubule-binding repeats. They also contain small amounts of MAP2b mRNA. Six low molecular weight tau isoforms, namely tau 1–6, have been described in the brain (Goedert et al. 1991). The major isoform of tau mRNA in oligodendrocytes was found to be tau 1, which represents a marker typical for immature neurons. Tau 2 and tau 4 isoforms were also detected, albeit at a very low level. Immunoblot analysis of oligodendroglia cell extracts confirmed the presence of tau protein. It migrates as a single polypeptide with an apparent molecular weight of approximately 55 kDa. In addition, oligodendrocytes express MAP2c protein, which migrates as a close double band with an apparent molecular weight around 70 kDa. Indirect immunofluorescence staining indicated that tau and MAP2 immunoreactivity was expressed in oligodendrocytes of immature and mature morphologies in the cell somata and cellular processes. Tau was particularly found in the end of the cellular extensions, and both proteins exhibited a distribution similar to myelin basic protein. Thus, oligodendroglia, like neuronal cells, contain microtubule-associated proteins, mainly MAP2c and the tau 1 isoform, although at a much lower level. The presence of these MAPs in myelin-forming cells further points to the functional significance of the cytoskeleton during oligodendrocyte differentiation, process outgrowth, and myelin formation.

Key words: Microtubules – MAP2 – Tau – Cytoskeleton – RT-PCR – Oligodendrocyte – Brain – Cell culture – Rat (Wistar)

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Introduction

During the active stage of myelination oligodendrocytes produce vast amounts of myelin membranes (for review see Pfeiffer et al. 1993). Oligodendrocytes in culture send out a multitude of cellular processes and elaborate large membranous sheets. These sheets contain an extensive cytoskeletal network of microtubules and microfilaments, which interact with myelin basic protein (MBP) and 2,3-cyclic nucleotide-3-phosphohydrolase (CNP; Wilson and Brophy 1989; Dyer and Benjamins 1989). While MBP is involved in myelin compaction, the physiological function of CNP is not yet clear (Pfeiffer et al. 1993). Oligodendrocytes appear to be devoid of intermediate filaments (Wilson and Brophy 1989; Pfeiffer et al. 1993). While immature cells possess many fine processes, which are especially rich in microfilaments, in differentiated large oligodendrocytes fewer and thicker microtubule containing processes are present (Wilson and Brophy 1989). The cytoskeleton in myelin-forming cells, and in particular the microtubules, play an important role during the extension and stabilization of myelin processes, and during myelin formation and compaction, and participate in the translocation of MBP mRNAs to the forming myelin sheath (Ainger et al. 1993; Brophy et al. 1993; Trapp et al. 1995).

Microtubules and their associated proteins (MAPs) have been most extensively studied in neuronal cells. MAPs have been implicated in the regulation of the stability of microtubules and are thought to contribute to the development of neuronal polarity (Matus 1994). The

most abundant MAPs in nerve cells are the two related proteins MAP2 and tau. Both proteins comprise a family of various isoforms, which are derived by alternative splicing of the product of a single copy gene, and bind to the walls of microtubules. The microtubule-binding domains consist of three or four repeats (3R; 4R) composed of homologous 18-amino-acid sequences, located near the carboxy terminus (for review see Goedert et al. 1991; Matus 1994). Three isoforms of MAP2 containing three tandem repeats for microtubule-binding domains (3R-MAP2) were identified on SDS polyacrylamide gels, namely MAP2a (290 kDa), MAP2b (280 kDa), and MAP2c (70 kDa), the latter being predominantly expressed in the developing brain (for review, see Goedert et al. 1991). Recently, a MAP2c isoform containing an additional microtubule-binding repeat (4R-2c) was found to be expressed in the adult rat brain and was suggested to play a role in extended neurites similar to that attributed to mature tau (Ferhat et al. 1994).

Tau proteins consist of two groups, the more recently discovered high molecular weight tau (HMW tau) which is preferentially expressed in the peripheral nervous system (Oblinger et al. 1991; Georgieff et al. 1991), and the low molecular weight tau isoforms (LMW tau) which are highly expressed in the CNS (Cleveland et al. 1979; Binder et al. 1985). Six LMW mRNAs (tau 1–6) encoding the LMW tau isoforms (A-F; 48–65 kDa) have been described (Goedert et al. 1991). Three of these LMW mRNAs contain an exon encoding an additional microtubule-binding motif (4R-tau), which is typically present in mature brain and is thought to contribute to enhanced microtubule stability (Goedert et al. 1991; Matus 1994). Thus, like MAP2, the tau family of proteins contains three or four homologous microtubule-binding domains, which are involved in the regulation of microtubule stability and rigidity, and hence contribute to neuronal specificity (Goedert et al. 1991; Matus 1994).

Little is known about the functional role of microtubule-associated proteins in the regulation of the oligodendrocyte cytoskeleton and the establishment of myelin-containing membranous sheets. In oligodendrocytes, the presence of MAP1B, a MAP with a widespread distribution, has been reported by several authors (Bloom et al. 1984; Fischer et al. 1990; Vouyiouklis and Brophy 1993). While MAP2a and b are suggested to be neuron specific and are also expressed by reactive astrocytes (Geisert et al. 1990), low levels of the MAP2c isoform with four microtubule-binding domains (4R-2c) are expressed in glial cell cultures (Doll et al. 1993; Ferhat et al. 1994). In the studies by Doll et al. (1993) and Ferhat et al. (1994) no discrimination between the two macroglia cell types, i.e., astrocytes and oligodendroglia, was made. The presence of MAP2b and MAP2c mRNA in astrocytic cultures, on the other hand, was shown by Northern blot experiments which could not distinguish between 3R and 4R isoforms (Charrière-Bertrand et al. 1991). The transient expression of MAP2c in developing rat brain oligodendrocytes in culture was described recently by Vouyiouklis and Brophy (1995). Regarding the expression of tau in oligodendrocytes, conflicting evidence has emerged. While Vouyiouklis and Brophy (1995) could not identify tau in their system, LoPresti et al. (1995) demonstrated the presence of tau mRNA by in situ hybridization and tau protein by means of immunohistochemistry and immunoblotting in oligodendrocytes in situ and in culture. However, so far, studies have not distinguished between the various isoforms.

To further examine the presence of MAP2 and tau in oligodendrocytes, we have prepared secondary cultures of highly purified rat brain oligodendrocytes and investigated the expression of alternatively spliced isoforms of mRNAs encoding MAP2 and tau by means of reverse transcription and polymerase chain reaction (RT-PCR) using a newly designed set of MAP2- and tau-specific primers. For the first time this made it possible to distinguish between the different tau mRNA isoforms in glial cells. Additionally, protein expression was assessed by immunoblot analysis and indirect immunofluorescence staining. Data were compared with the mRNA and protein expression patterns of MAP2 and tau in cultures of rat brain astrocytes and rat cerebral neurons, and with adult rat brain tissue. The present study demonstrates that oligodendroglia express MAP2, mainly MAP2c, and also at least two different isoforms of LMW tau.

Materials and methods

Materials

Cell culture media were from Gibco/BRL (Grand Island, NY). Monoclonal antibodies against tau (clone no. tau-2) and MAP2 (clone no. HM-2), recognizing all tau- and MAP2- isoforms, were from Sigma (St. Louis, MO). Polyclonal rabbit antisera against horseradish-peroxidase (HRP)-, fluorescein-isothiocyanate (FITC)-, and rhodamine-isothiocyanate (RITC)-conjugated secondary antibodies were from Sigma (St. Louis, MO). Polyclonal rabbit anti-MBP antiserum was from J.-M. Matthieu (Matthieu and Amiguet 1990).

Cell culture

All cells were kept at 37°C and 10% CO_2. Growth medium was changed three times a week.

Primary glial cells. Primary cultures of glial cells were prepared from the brains of 1- to 2-day-old Wistar rats. Cerebral hemispheres were freed of the meninges and mechanically disrupted using a Pasteur pipette. Single cell suspensions were transferred to culture flasks (1 brain/75 cm^2) and kept for 6–8 days in DMEM supplemented with 10% heat-inactivated fetal calf serum (FCS), 50 U/ml penicillin, and 50 µg/ml streptomycin. For Western blot analysis and RNA preparation, secondary cultures containing either oligodendrocytes or astrocytes were prepared from the flasks after 6–8 days as described previously (Richter-Landsberg and Heinrich 1995). Briefly, the phase dark cells growing on the astrocytic cell layer were separated by vigorous shaking and taken off as described by McCarthy and DeVellis (1980). The cells floating on top of the astrocytic cell layer were replated on poly-L-lysine-coated culture dishes (10^6 cells/60 mm dish) and incubated in serum-free DMEM supplemented with insulin, (5 µg/ml) transferrin, (5 µg/ml), and sodium selenite (25 ng/ml; Boehringer, Mannheim). After 1 week, these cultures contained a highly enriched population of differentiated oligodendrocytes with numerous cellular processes (Richter-Landsberg and Heinrich 1995). The re-

maining astrocytes were subcultured in uncoated 10-cm culture dishes for 3–4 weeks in DMEM supplemented with 10% FCS (Richter-Landsberg and Besser 1994).

Primary neuronal cells. Single-cell suspensions were prepared from cerebral hemispheres of 16-day-old rat embryos as described previously (Richter-Landsberg 1988). Cells were kept in Eagles basal medium (BME), containing 0.5% FCS for 10 days.

Indirect immunofluorescence staining

For fluorescent labeling experiments cells were grown on poly-L-lysine-coated glass coverslips (100 000 cells per 35-mm dish). After washing with PBS, cells were fixed with 3% paraformaldehyde for 10 min. For intracellular staining paraformaldehyde-fixed cells were pretreated with 0.1% Triton X-100 containing 2% FCS (30 min). Thereafter, cells were incubated with the first antibodies for 60 min. The antibodies were used at the following dilutions: anti-tau, 1:100; anti-MAP2, 1:100; anti-MBP, 1:100. After washing with PBS, cells were incubated with FITC-conjugated goat anti-mouse IgG (1:75) or anti-rabbit IgG (1:75) for 45 min, washed with PBS, and mounted. For double immunofluorescent staining the first and second stainings were performed in sequence, and RITC-conjugated anti-rabbit IgG (1:75) was used as the secondary antibody. Fluorescent labeling was examined using a Zeiss epifluorescence microscope equipped with an automatic camera using a plan-neofluar objective (40×). Control experiments, using the secondary antibodies only, did not reveal unspecific staining.

Western blot analysis

Cellular monolayers were washed with PBS three times, scraped off in sample buffer (Laemmli 1970) containing 1% SDS and boiled for 10 min. Protein contents in the samples were determined according to Neuhoff et al. (1979). For immunoblotting, total cellular extracts (10 µg protein per lane, if not indicated otherwise) were separated by one-dimensional SDS-PAGE using 10% polyacrylamide gels or as indicated, and transferred to nitrocellulose membranes (Schleicher and Schuell, Dassel/Germany; 0.2 µm) according to Towbin et al. (1979). The blots were washed and incubated with the appropriate first antibodies (anti-tau 1:1000; anti-MAP2 1:1000) followed by HRP-conjugated anti-mouse IgG (1:5000) and visualized by the enhanced chemiluminescence (ECL) procedure as described by the manufacturer (Amersham, Braunschweig).

RNA extraction and reverse transcription

RNA was isolated by a modification of the method described by Chomczynski and Sacchi (1987). Briefly, rat brain tissue or cells were homogenized in guanidinium thiocyanate solution and repeatedly extracted with water-saturated phenol. After extraction with chloroform, RNA was precipitated by isopropanol, collected by centrifugation and dissolved in water treated with diethylpyrocarbonate (DEPC). RNA was reprecipitated by addition of LiCl and ethanol, dissolved in DEPC-treated water and quantified by spectrophotometry. One microgram of RNA was used for reverse transcription in a final volume of 20 µl. First strand synthesis conditions were: 50 mM TRIS-HCl pH 8.3, 50 mM KCl, 10 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM spermidine, 0.25 mM of each dNTP, 12.5 pmol each of oligo-dT_{16} and random hexamer primers, and 5–8 U reverse transcriptase AMV (avian myeloblastosisvirus) AMV (Promega, Madison, USA). After incubation at 42°C for 1 h, the reaction mixture was diluted to 100 µl with water and stored at –80°C. Subsequently, 1–3 µl were used for PCR analysis. In addition to the cDNA prepared from adult rat brain tissue, 1 µl of a cDNA library from adult rat brain (937502, Stratagene, La Jolla, USA) was used in some experiments.

PCR and primers

Primers were synthesized by Pharmacia (Freiburg, Germany) and designed using the PrimerSelect software (DNASTAR, Madison, USA).

The following primers were used for analysis of MAP2: 2b1s, GCCTGTGGGTGGACACTCAAG (4237–4257, MAP2b); 2b2as, TGACGTCCTCAGCCAAAGTGG (5503–5523, MAP2b; 1416–1436, MAP2c); and 2c3s, GGTCACAGGGCACCTATTCAG (244–264, MAP2b and MAP2c).

Numbers in brackets indicate the positions of primers in the rat MAP2b and 2c sequences as published by Kindler et al. (1990).

For the analysis of tau the following oligonucleotides were used: t1, CGCCAGGAGTTTGACACAATG (52–72); t2, GCCCTTGGCTTTCTTCTCGTC (1180–1200); t3, GAACGAAGCGGCTACAGCAGC, (1351–1371); and t4, GAGATGTGTCCCCAGACACCA (1967–1987).

Numbering is according to the sequence of rat tau from the peripheral nervous system (Goedert et al. 1992).

Control experiments were carried out with the following primers: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), CCCACGGCAAGTTCAACGGCA (220–240) and TGGCAGGTTTCTCCAGGCGGC (805–825; Fort et al. 1985); MBP, GACCCTCACAGCGACACGGAT (41–61) and CTGCTGAGGGACAGGCCTCTC (336–356; Roach et al. 1983), glial fibrillary acidic protein (GFAP), ACCATGCCACGTTTCTCCTTGTCTCG (7–32) and CCTTAATGACCTCACCATCCCGCATCT (1151–1177; Lewis et al. 1984); and neurofilament middle molecular weight (NF-M), GAAATCAAGGTCGAGAAGCC (1783–1803) and CTAAGCCATTAGTGACCACCC (2300–2279; Napolitano et al. 1987).

PCR reactions were carried out in a volume of 23 µl containing 10 mM TRIS-HCl, pH 9.0, 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM of each dNTP, and 0.8 µM of each primer. After an initial denaturation for 2 min at 95°C, 2 µl of Taq polymerase (0.5 U/µl) was added. In a Varius V 45 cycler (Landgraf, Langenhagen, Germany) 25–35 cycles were performed, each consisting of a 1-min denaturation at 95°C, 1-min primer annealing at 56°–60°C, depending on the primer pair used, and a 2-min elongation at 72°C. After a terminal extension for 5 min at 72°C, reactions were stored at 4°C. For analysis by agarose gel electrophoresis 5–10 µl was used. Southern blots were performed using PCR products internally labeled with digoxigenin-UTP according to the manufacturer's instructions (Boehringer, Mannheim, Germany).

Sequencing

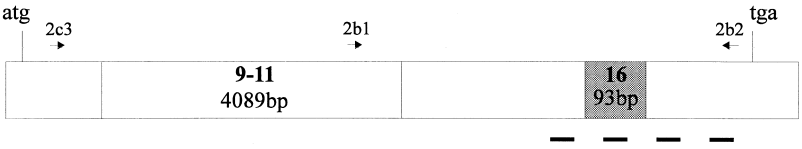
PCR products for sequencing were isolated from agarose gels with the "Qiaex Gel Extraction Kit" (Qiagen, Hilden) according to the procedure described by the manufacturer. Sequencing was done with an ABI 373A automated sequencer using PCR primers and fluorescent-labeled desoxy terminators according to the manufacturer's instructions (Applied Biosystems, Weiterstadt, Germany).

Results

Expression of MAP2 mRNA

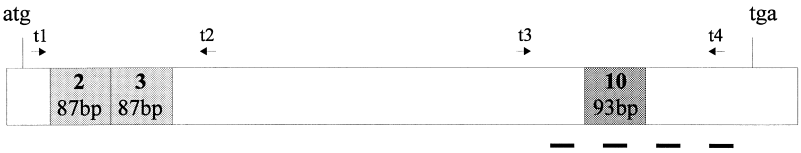
Total RNA was extracted from cultured oligodendrocytes. For this, oligodendrocytes were separated from astrocytes by mechanically shaking after 6 days and subcultured for another week. An RT-PCR analysis was used to identify the alternatively spliced products of the

a



mRNA	exons 9-11	exon 16	primers	product length
4R-MAP2b	+	+	2b1, 2b2	1380bp
3R-MAP2b	+	-	2b1, 2b2	1287bp
4R-MAP2c	-	+	2c3, 2b2	1286bp
3R-MAP2c	-	-	2c3, 2b2	1193bp

b



mRNA	exon 2	exon 3	exon 10	primers	product length
tau6	+	+	+	t1, t4	1174bp
tau5	+	-	+	t1, t4	1087bp
tau4	-	-	+	t1, t4	1000bp
tau3	+	+	-	t1, t4	1081bp
tau2	+	-	-	t1, t4	994bp
tau1	-	-	-	t1, t4	907bp
4R-tau			+	t3,t4	637bp
3R-tau			-	t3,t4	544bp
tau3/tau6	+	+		t1,t2	387bp
tau2/tau5	+	-		t1,t2	300bp
tau1/tau4	-	-		t1,t2	213bp

Fig. 1a, b. Schematic structure of the MAP2 and tau genes and composition of mRNA isoforms. The schematic structures of the MAP2 (**a**) and tau gene (**b**) including the position of alternatively spliced exons are symbolized by the *open* and *shaded boxes*. Alternative exons are numbered in *bold*, according to the human MAP2 gene (Kalcheva et al. 1995) and bovine tau gene (Himmler 1989). Their length is given in *normal type*. The translational start (*atg*) and stop codons (*tga*) as well as directions and names of primers used for RT-PCR are indicated above the gene structure. *Horizontal bars* indicate the position of the four microtubule-binding domains. The table shows the nomenclature and presence (+) or absence (–) of the alternatively spliced exons in different MAP2 mRNA isoforms (**a**) and tau mRNA isoforms (**b**) in addition to the combinations of primers used for analysis and the length of the respective PCR product. In cases where the primer combination cannot distinguish between the presence or absence of an exon, an *empty box* is given

MAP2 gene. In Fig. 1a a schematic representation of the MAP2 gene and the positions of the three oligonucleotide primers is depicted. These primers were selected to yield and differentiate between PCR amplification products of MAP2b and MAP2c with either three of four microtubule-binding repeats. The same primers were used to investigate mRNA expression in extracts prepared from rat cerebral cultures (10 days in vitro), cultures of 5-week-old astrocytes, and adult rat brain.

As seen in Fig. 2, the major PCR amplification products found in oligodendrocyte cell extracts represent the two MAP2c isoforms containing three and four microtubule-binding motifs (3R-2c and 4R-2c; Fig. 2b). Additionally, a MAP2b isoform with three microtubule-binding domains (3R-2b) was detected, albeit at a lower level (Fig. 2a). While this 3R-MAP2b isoform was also prevalently expressed in primary neuronal cultures and adult rat brain, cultured astrocytes contained only the MAP2b isoform with four microtubule-binding motifs (4R-2b), which was barely detectable in the other cell extracts and in the adult brain (Fig. 2a). The two MAP2c isoforms 4R-2c and 3R-2c, present in oligodendroglia, were also found in adult rat brain, whereas neuronal cells expressed mainly 3R-MAP2c, and astrocytes, the 4R-MAP2c isoform (Fig. 2b). The identity of the PCR products was analyzed by Southern blotting and confirmed by direct sequencing of the products eluted from the gels (data not shown).

Expression of tau mRNA

Four primers were used to identify tau mRNA isoforms. The schematic structure of the tau gene and the positions

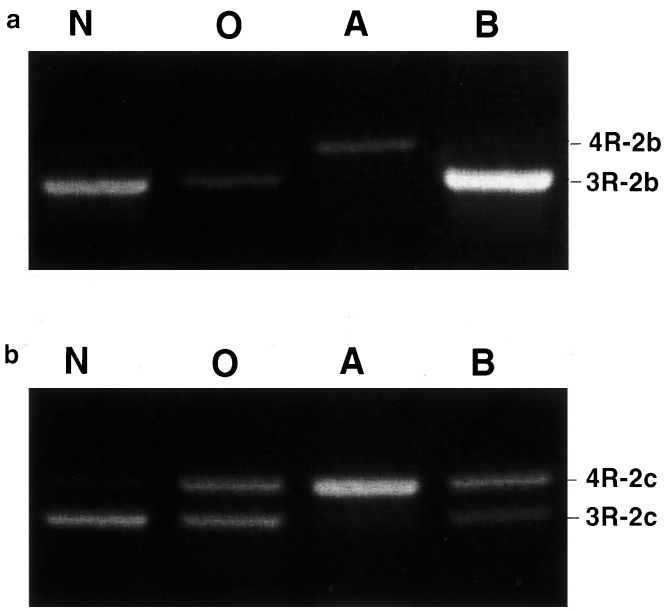


Fig. 2a, b. RT-PCR analysis of MAP2 mRNA isoforms. **a** Analysis of MAP2b mRNA expression using primers 2b1 and 2b2. **b** Analysis of MAP2c mRNA expression using primers 2b2 and 2c3. Letters on top indicate cell cultures used for RNA preparation: *N*, neurons; *O*, oligodendrocytes; *A*, astrocytes; *B*, Adult rat brain. PCR products representing MAP2 mRNA isoforms are marked on the *right*

of the primers in relation to the alternatively spliced exons is depicted in Fig. 1b. For RT-PCR analysis the same extracts as mentioned above were used and amplification products were identified by Southern blotting and direct sequencing (data not shown).

Using the outermost primer pair (t1 and t4) for PCR amplification, one major product corresponding to the tau-1 isoform and another rather faint band, representing tau-2 and/or tau-4 isoforms (tau 2/tau 4) could be identified in the oligodendrocyte cell extract (Fig. 3a). The RT-PCR product corresponding to tau 1 was also the major form expressed in neuronal cultures but could neither be found in astrocytes nor in adult rat brain (Fig. 3a). Amplification products corresponding to tau 2/tau 4 were detected in all cell extracts investigated and were most prominently expressed in adult rat brain (Fig. 3a). In addition, adult rat brain contained two bands, indicative of tau 6 and tau 3/tau 5 (Fig. 3a). Thus, oligodendrocytes and primary neuronal cells expressed a similar pattern of tau isoforms.

The similar length of exon 3 (87 bp) and exon 10 (93 bp) prevented the differentiation of the isoforms tau 2 and tau 4, and tau 3 and tau 5 (Fig. 1b) in the experiment just described. Therefore, two additional combinations of oligonucleotide primers were used to further investigate the presence of the alternatively spliced exons.

To investigate the presence of isoforms with exon 10, which encodes an additional microtubule-binding domain, t3 and t4 were used as primers (Fig. 1 b). RT-PCR revealed that oligodendrocytes and neurons expressed both products, i.e., the isoform with exon 10 (4R-tau) and the isoform without exon 10 (3R-tau; Fig. 3b).

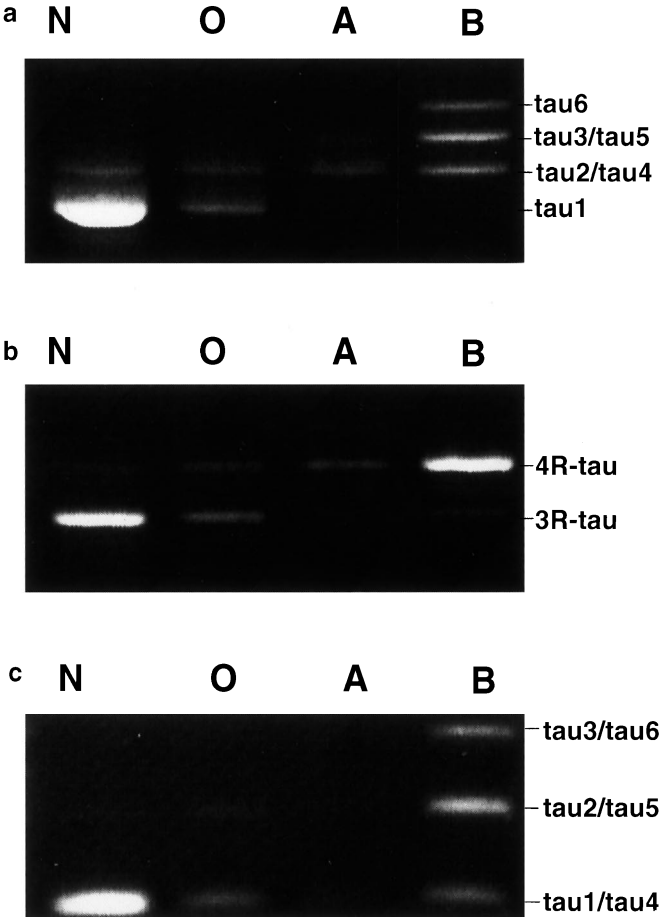


Fig. 3a–c. RT-PCR analysis of tau mRNA isoforms. **a** Analysis of tau mRNA expression using primers t1 and t4. **b** Analysis of the presence of exon 16 in tau mRNA using primers t3 and t4. **c** Analysis of the presence of exons 2 and 3 in tau mRNA using primers t1 and t2. Letters on top indicate cell cultures used for RNA preparation: *N*, neurons; *O*, oligodendrocytes; *A*, astrocytes. *B*, Adult rat brain. PCR products representing tau mRNA isoforms are marked on the *right*

While astrocytes contain only the 4R-tau isoform, both isoforms were also identified in rat brain. However, compared with primary neuronal cultures with the 3R-tau isoform being most prominent, in adult rat brain the 4R-tau isoform containing exon 10 was much more abundant (Fig. 3b). In oligodendrocytes the 3R-tau isoform was most prominently expressed.

Using primers t1 and t2 as a third combination, we investigated the occurrence of exon-2- and exon-3-containing isoforms (Fig. 1b). Adult rat brain expressed all three possible splicing variants in similar amounts (Fig. 3c). Analysis of oligodendrocytes and neuronal cells showed the presence of tau 1/tau 4 mRNA lacking exons 2 and 3 and a rather faint expression of tau 2/tau 5 mRNA missing only exon 3 (Fig. 3c). Only the former component was also seen in astrocytic cell extracts, although at a low level (Fig. 3c). Thus, oligodendrocytes and neuronal cells expressed mainly the tau isoforms lacking exons 2 and 3.

In summary, oligodendrocytic cell extracts contained mainly the tau-1 isoform missing all of the alternatively

spliced exons, and smaller amounts of tau2 and tau4 mRNA. A similar pattern was found in primary neuronal cells.

Expression of GAPDH and neuron- and glial-specific mRNAs

To control for equal loading of cDNA amounts during the experiments, RT-PCR analysis of GAPDH expression was carried out (Fig. 4a). Additionally, to estimate the purity of the individual culture systems used in this study, RT-PCR was performed using primers to amplify cDNAs encoding the neuron-specific middle molecular weight (140 kDa) NF-M, the astrocyte-specific intermediate filament protein GFAP, and MBP, which is only present in oligodendrocytes. The PCR product of NF-M could only be detected in neuronal cell extracts and rat brain, and was not present in oligodendrocytes or astrocytes (Fig. 4b). To detect cDNA encoding MBP, we used primers from exon 1 and exon 4 of the MBP gene, which yields two amplification products with or without exon 2 (Roach et al. 1983; Martenson 1992). Both products were prominently expressed in oligodendrocytes and adult rat brain, but were absent in neuronal cultures, and only the lower molecular weight product was faintly expressed in primary astrocytic cultures (Fig. 4c). The PCR amplification product for GFAP was most prominent in primary astrocytes but also detectable in other cell extracts, indicating the presence of astrocytes in all of the culture systems investigated (Fig. 4d).

Immunoblot analysis of MAP2 and tau proteins

The presence of MAP2 and tau proteins in oligodendrocyte cell extracts was investigated by Western blot analysis. Using monoclonal antibodies which distinguish between all isoforms of MAP2, it was found that oligodendrocytes express only the low molecular weight form of MAP2, i.e., a 70-kDa component (MAP2c) resolved as a closely migrating double band, most likely representing differentially phosphorylated polypeptides (Goedert et al. 1991; Fig. 5). While MAP2c was also the only isoform detectable in astrocyte cell extracts, neuronal cultures additionally contained the high molecular weight 280-kDa MAP2b isoform (Fig. 5).

Using monoclonal antibodies directed to all tau isoforms, an immunoreactive band with an apparent molecular weight of approximately 55 kDa was resolved in the immunoblots of the oligodendroglial cell extract (Fig. 6). This component was also seen in neuronal cultures and most prominently expressed in cell extracts prepared from newborn rat brain (Fig. 6).

Immunofluorescence analysis of MAP2 and tau in oligodendroglia

Indirect immunofluorescence studies demonstrated the presence of MAP2 and tau proteins in oligodendroglia. Using monoclonal anti-MAP2 antibodies, recognizing

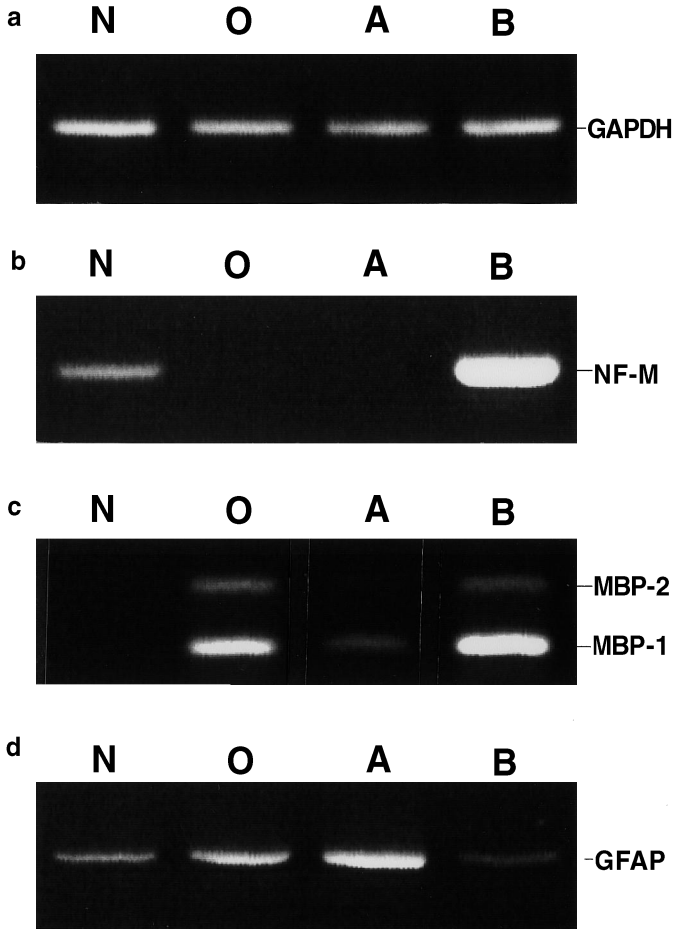


Fig. 4a–d. RT-PCR analysis of GAPDH and neuronal- and glial-specific mRNAs. **a** Analysis of *GAPDH* mRNA expression. **b** Analysis of *NF-M* mRNA expression. **c** Analysis of *MBP* mRNA expression. *MBP-1* and *MBP-2* denote alternatively spliced mRNAs with and without exon 2, respectively. **d** Analysis of *GFAP* mRNA expression. Letters on top indicate cell cultures used for RNA preparation: *N*, neurons; *O*, oligodendrocytes; *A*, astrocytes. *B*, Adult rat brain. PCR products representing the different mRNAs are marked on the *right*

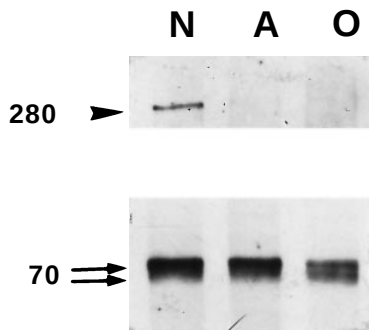


Fig. 5. Immunoblot analysis of MAP2 protein in cell extracts. Cell extracts of oligodendrocytes (*O*) were subjected to Western blot analysis and compared with cell extracts of cultured embryonic neurons which were kept for 10 days in vitro (*N*) and cultured astrocytes which were kept for 35 days in vitro (*A*). Ten micrograms of each cell extract was loaded. *Numbers* on the *left* represent molecular weights in kDa. To identify the 280-kDa MAP2 (*arrowhead*) a 5% polyacrylamide gel was used (*upper panel*), and the 70-kDa MAP2 (*double arrow*) was analyzed on a 10% gel

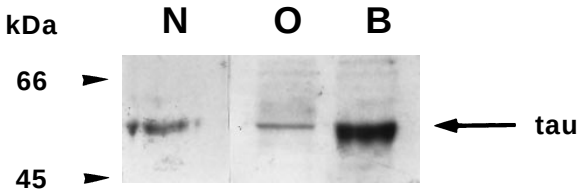


Fig. 6. Immunoblot analysis of tau protein in cell extracts. Cell extracts of oligodendrocytes (11 days in vitro; *O*) were subjected to Western blot analysis and compared with cultured embryonic neurons which were kept for 10 days in vitro (*N*) and brain of newborn rat (*B*). *Arrow* points to the 55-kDa tau-immunoreactive polypeptide. Thirty micrograms of oligodendroglia cell extracts, 20 μg of neuronal cell extracts, and 10 μg of brain extract was loaded onto the gels

all isoforms, MAP2 immunoreactivity was found in the small cell bodies and cellular processes of oligodendrocytes with a distribution similar to that of MBP (Fig. 7). Immunofluorescent staining of tau, using monoclonal antibodies against all isoforms, was detected in the cell somata and cellular processes of oligodendrocytes of mature and rather immature morphology (Fig. 8 b–d). Tau immunoreactivity seemed to be enriched in the tips of the cellular processes and generally had a distribution similar to that of MBP (Fig. 8 a,b).

Discussion

Microtubule-associated proteins play an important role in the promotion of microtubule assembly and stabiliza-

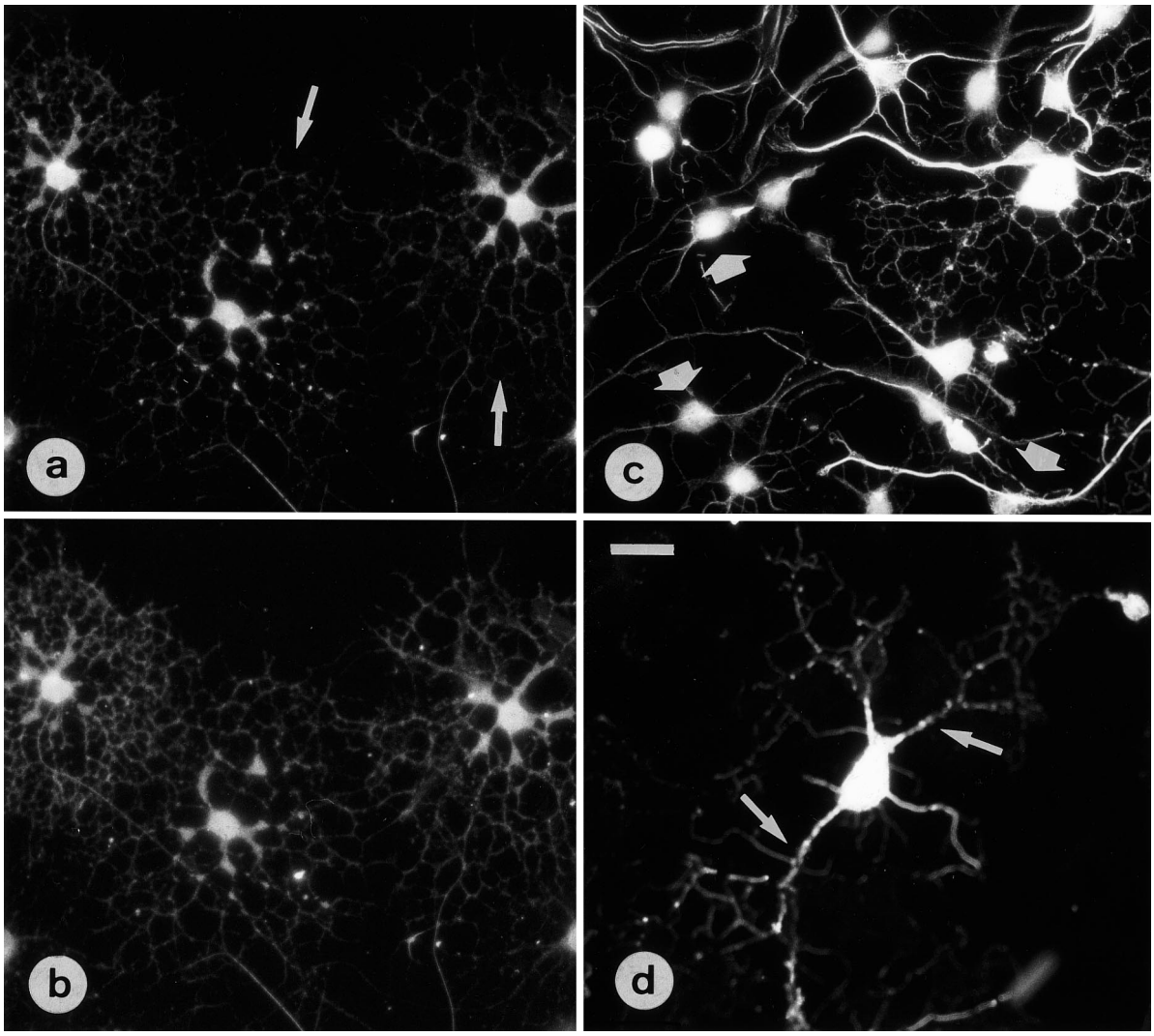


Fig. 7a–d. Immunofluorescence analysis of the distribution of MAP2 protein in oligodendrocytes. Oligodendrocytes were separated from the astrocytes by mechanically shaking and subcultured on poly-L-lysine-coated coverslips for 5 days. Immunofluorescence staining was carried out with anti-MAP2 (**a, c, d**) and anti-MBP (**b**) antibodies. Double immunofluorescent staining was carried out in sequence: a group of multiprocessed oligodendrocytes stained with anti-MAP2 (**a**) and the same cells stained with anti-MBP (**b**) are shown. MAP2 immunoreactivity was observed in the cell somata of immature (**c**, *arrows*) and mature (**a, d**) oligodendrocytes, where the primary processes are heavily stained (**d**, *arrows*). The fine cellular processes (**a**, *arrows*) contain MAP2 in a distribution similar to that of MBP (**b**). The *bar* represents 25 μm

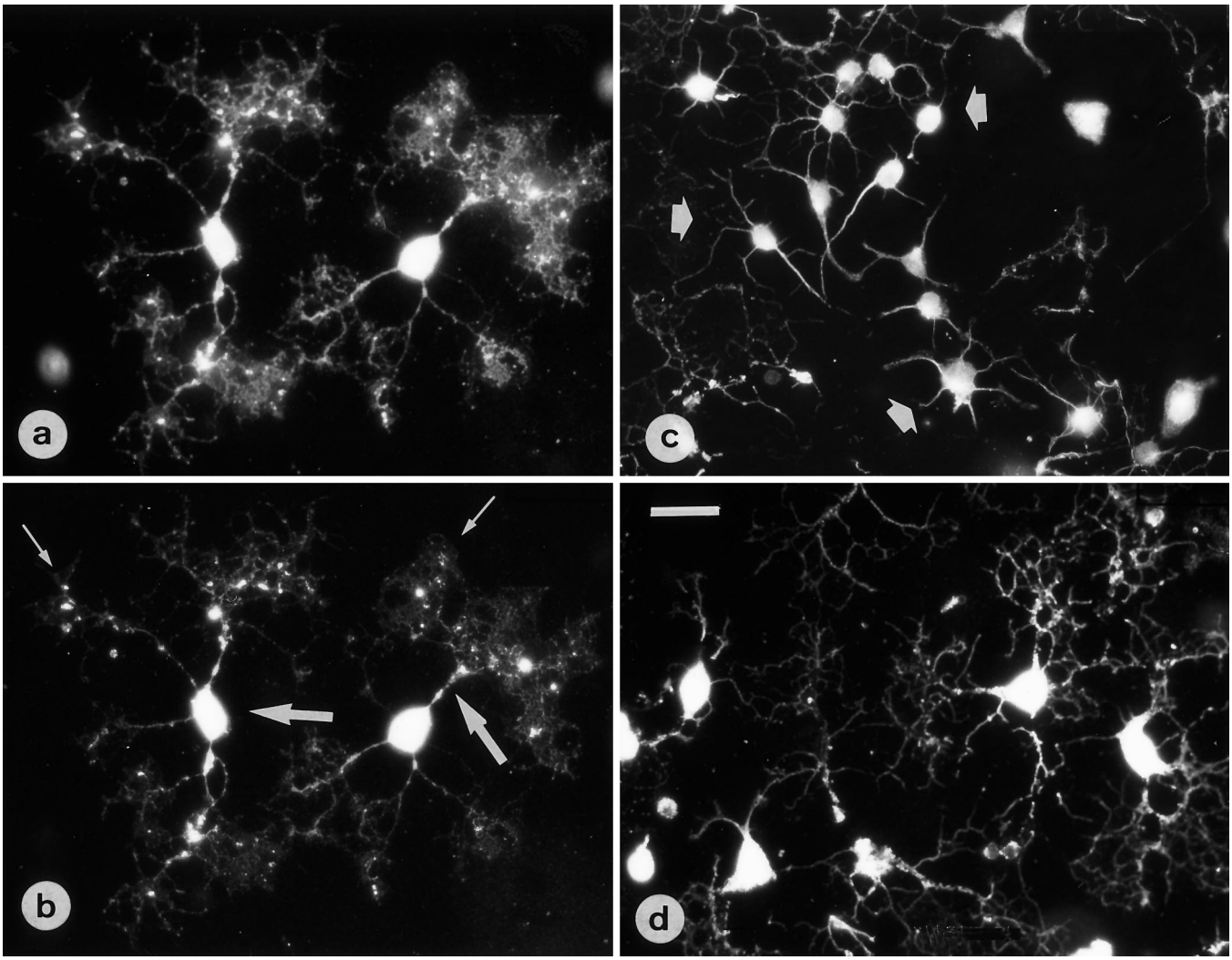


Fig. 8a–d. Immunofluorescence analysis of the distribution of tau in cultured oligodendrocytes 5 days after subplating. Indirect immunofluorescence staining was carried out with anti-tau (**b, c, d**) and anti-MBP (**a**) antibodies. Double immunofluorescence with anti-MBP and anti-tau was carried out in sequence: two large oligodendrocytes with a mature morphology stained with anti-MBP antibody (**a**) and the same cells stained with anti-tau antibody (**b**) are shown. Tau immunostaining is present in the cell body and primary processes (**b**, *large arrows*) and also in the tips of the cellular processes (**b**, *small arrows*). Tau immunoreactivity was abundant in the cell somata and cellular processes of oligodendrocytes with immature morphology (**c**, *arrows*), in cells with numerous processes (**d**), and in mature cells (**b**). The *bar* represents 25 μm

tion. During brain development they have been shown to undergo marked changes in composition (Riederer and Matus 1985; Goedert et al. 1991). They are most abundantly expressed in nerve cells and are involved in the determination of neuronal shape and plasticity. Oligodendrocytes in culture are characterized by large membranous sheets containing a network of microtubules, which are concentrated in the major veins (Dyer and Benjamins 1989). This elaborate cytoskeletal network is not only essential for the elaboration and maintenance of the myelin-containing cellular processes but also plays a major role in intracellular sorting and transport (Brophy et al. 1993). Thus, the analysis of the cytoskeleton and its associated proteins, which participate in microtubule stability and regulation of the cellular architecture, contributes to the elucidation of the specific mechanisms leading to myelin formation.

The present study shows that the two major groups of neuronal microtubule-associated proteins, MAP2 and tau, are also expressed in the myelin-forming cells of the CNS. While the high molecular weight (HMW) MAP2a and MAP2b forms are mainly considered as neuron-specific and are highly concentrated in neuronal dendrites (Goedert et al. 1991), MAP2c has previously been also localized in glial cells. For instance, Tucker et al. (1988) detected MAP2c in Bergman glia cells. As found by Northern blotting, MAP2c was also the dominant MAP2 transcript in cultured astrocytes prepared from mouse brain; HMW MAP2 was found to be present in small amounts in these cells (Charrière-Bertrand et al. 1991). Using reaggregated rat brain cell cultures and immunoblot analysis, MAP2c with four microtubule binding domains (4R-2c) was found to be associated with glial cells, while MAP2c with three microtubule

binding domains (3R-2c) seemed to be expressed in neurons (Doll et al. 1993). However, in the study by Doll et al. (1993) cultures were treated with cytotoxic drugs in order to eliminate dividing glial cells, and no discrimination between the two types of macroglial cells, i.e., astrocytes and oligodendrocytes, was made. Similarly, Ferhat et al. (1994), using RT-PCR, detected small amounts of mRNA encoding 4R-MAP2c in secondary glial cultures.

In a recent study, Vouyiouklis and Brophy (1995) demonstrated that oligodendrocytes in culture express MAP2. On the basis of RT-PCR experiments using two primer pairs, flanking nucleotide 4089 of the sequence specific for MAP2b and exon 16 encoding the fourth microtubule-binding domain, respectively, they suggested that oligodendrocytes exclusively express 4R-MAP2c.

Our study, using cultures of highly enriched rat brain oligodendrocytes which, as estimated by indirect immunofluorescence analysis and phase-contrast microscopy, contain less than 10% contaminating astrocytes (Richter-Landsberg and Heinrich 1995), and cultures of type 1 astrocytes, demonstrates that in oligodendrocytes 3R- and 4R-MAP2c transcripts are present. In contrast, astrocytes predominantly express 4R-MAP2c and neuronal cells, only the 3R-MAP2c transcript. Our control experiments demonstrate that all culture systems contain small amounts of astrocytes, but neuronal cells are not present in the glial cultures (Fig. 4). The 4R-MAP2c transcript found in oligodendrocyte cell extracts might therefore result from astroglia contamination, but the 3R-MAP2c transcript clearly can be attributed to oligodendroglial cells.

3R-MAP2b is prominently expressed in neuronal cells and rat brain. While Vouyiouklis and Brophy (1995) did not identify this isoform, we could detect small amounts of 3R-MAP2b transcripts in oligodendroglia cell extracts. This finding might be explained by the specificity or different positions of the primers used in both studies. We have analyzed MAP2b expression with one primer inside the MAP2b specific exon 9, yielding an amplification product of 1287 bp. Vouyiouklis and Brophy (1995) used a primer pair flanking the complete sequence of exons 9–11, which simultaneously amplifies cDNA derived from MAP2b and MAP2c. Thus, during the PCR-amplification procedure the >4-kb-long PCR product encoding MAP 2b might be generated in a much lower amount than the 0.3-kb MAP2c-specific PCR product.

Interestingly, in astrocytic cell extracts we could identify 4R-MAP2b mRNA. This isoform has been described so far only in a neuronal cell line (Langkopf et al. 1994) and only very low levels are present in the adult brain (Doll et al. 1993; Kindler and Garner 1994).

An immunoblot analysis further corroborated the presence of MAP2c protein in the cellular extracts of all three cell culture systems. We could not detect HMW MAP protein in glial cells, which might be explained by the rather low level of the mRNA as seen by RT-PCR or the fact that MAP2b expression is also regulated post-transcriptionally (Charrière-Bertrand et al. 1991).

Thus, oligodendroglia, like neuronal cells express the 3R-MAP2c transcript, which encodes a MAP2 isoform with a potential functional role in maintaining the plasticity of the microtubule network during early times of development (Doll et al. 1993; Ferhat et al. 1994). The 4R-MAP2c expression, which continuously increases during postnatal brain development (Doll et al. 1993), seems to be mainly confined to astrocytes.

Tau proteins promote microtubule assembly and stability in the nervous system, and the binding of tau reduces the dynamic instability of microtubules (for review, see Goedert 1993). In immature brain, only the tau isoforms with three microtubule-binding domains are expressed, while isoforms with four repeats are adult specific (Goedert et al. 1989a,b) and are more potent in promoting tubulin polymerization in vitro (Goedert and Jakes 1990). Expression of tau mRNA in cultured astrocytes was investigated by Couchie et al. (1988). The presence of tau protein in oligodendrocytes was recently described by LoPresti et al. (1995). In this study tau was identified by immunostaining, in situ hybridization, and Western blot analysis in rat brain sections and as well as in cultures derived from lamb brain. Using primary cultures of rat brain, Vouyiouklis and Brophy (1995) could not detect tau expression in oligodendrocytes at any developmental stage.

The data presented here demonstrate that oligodendrocytic cell extracts contain tau mRNA with three and four microtubule-binding repeats and that the major isoform of tau mRNA expressed in oligodendrocytes is tau 1. Additionally, small amounts of tau 2 and tau 4 mRNA are also detected (Fig. 3). All three isoforms are present in neuronal cell cultures, but tau 1 was most prominently expressed. Tau 1 represents a typical marker for immature neurons (Kosik et al. 1989; Goedert et al. 1991) and is completely absent from astroglial cells (Fig. 3). In astroglia cell extracts only 4R-tau isoforms are found, especially tau 4, albeit at a rather low level. Thus, tau 1 and tau 2 mRNA may be specific for oligodendroglial cells, whereas the presence of tau 4 mRNA might result from contaminating astrocytes present in this system (see above). Immunoblot analysis showed a distinct tau-immunoreactive polypeptide band of approximately 55 kDa, and indirect immunofluorescence staining revealed that tau immunoreactivity is present within the cell somata and along the processes with a distribution similar to that of MBP and a rather punctuated staining pattern. Thus, like nerve cells, oligodendrocytes express tau protein, but to a much lower extent.

Tau proteins are phosphorylated at multiple sites, which contributes to the complex pattern of tau bands. Phosphorylation of tau reduces its ability to bind to microtubules (Matus 1994). The developmental regulation and dynamic balance of tau phosphorylation and dephosphorylation is essential for normal development and is disturbed in Alzheimer's disease (Goedert 1993; Garver et al. 1996). Its implication for oligodendrocyte differentiation and process formation remains to be established. As shown by LoPresti et al. (1995) and also in this study, tau in oligodendrocytes appears to colocalize with MBP, which is located in the intracellular membrane domains. In a number of studies, MBP was suggested to regulate cytoskeletal assembly and stability in

oligodendrocytes by mediating extracellular signals using a transduction mechanism which most likely includes MBP-phosphorylation (Dyer et al. 1994, 1995). Thus, tau protein might participate in the MBP-mediated transmembrane signaling events which possibly regulate various aspects of cytoskeleton assembly in myelin-forming cells.

To conclude, the presence of MAP2 and tau proteins, in addition to the previously described MAP1B (Fischer et al. 1990; Vouyiouklis and Brophy 1993), further points to the functional significance of the cytoskeleton during oligodendrocyte differentiation, process outgrowth, and myelin formation. The molecular mechanisms underlying these processes are highly specific, and similarly to their well-established roles in neuronal cells, MAPs in oligodendrocytes might provide the structural stability and required plasticity of the oligodendroglial cytoskeleton.

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