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Developmental Expression of Myelin Proteolipid, Basic Protein, and 2′,3′-Cyclic Nucleotide 3′-Phosphodiesterase Transcripts in Different Rat Brain Regions

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Abstract. RNA was extracted from five different rat brain regions during development, starting from embryonic day 15 (E_{15}) until postnatal day 60 (P_{60}). These RNA preparations were analyzed by both Northern and dot blot for their content of 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase), myelin proteolipid protein (PLP), and myelin basic protein (MBP) -specific transcripts. CNPase mRNA was readily detectable at E_{15} and PLP mRNA at P_1 in all brain regions examined. In contrast, expression of MBP mRNA followed a caudorostral gradient. It was first observed at P_1 in the mesencephalon and at P_9–P_{11} in the olfactory bulb. Expression of these three transcripts displayed two types of developmental profiles. One was termed biphasic because the specific mRNA level increased regularly and then reached a plateau level. The other developmental profile was termed triphasic, because there was a gradual increase in the level of specific transcripts with a sudden appearance of a sharp peak followed by a decline to a plateau level. When the triphasic pattern was observed, the date of the peak appearance was probe-, but not region-, dependent. It was P_{15} for CNPase, P_{18} for MBP, and P_{21} for PLP. As these peaks occurred at a time during development when myelination was the most active, we postulate the existence of a transient external signal, perhaps neuronal, which would be responsible for this increased amount of myelin-related transcripts.

Central nervous system myelin is synthesized by the oligodendrocytes. The work of Privat and Leblond (1972) and Paterson et al. (1973) and, more recently, the studies conducted by Raff et al. (1983, 1985) and by Levi et al. (1986) have determined the ontogenic origin of oligodendrocytes. The order of immunodetectability of four myelin markers, galactosylceramide (GalC), Wolfgram protein (W_1), myelin basic protein (MBP), and proteolipid protein (PLP), was examined prior to and during the period of early myelinogenesis in the mouse (Monge et al., 1986). It was observed that during development, myelin constituents are synthesized by the oligodendrocytes at two intervals separated by a five-day time lag. The early appearance of GalC and W_1 occurs just after differentiation of the progenitor cell when the oligodendrocyte is still undergoing cell division. A postmitotic late appearance of MBP and PLP occurs shortly before the oligodendrocyte begins to myelinate axons. Similar results were reported from in vitro experiments by Dubois-Dalcq

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Fig. 1. Comparative Northern analysis of CNPase, MBP, and PLP transcripts during development in different rat brain region. Aliquots (15 μg) of total RNA isolated from rat olfactory bulb (1A), cerebral hemispheres (1B), diencephalon (1C), cerebellum (1D), and mesencephalon (1E) of postnatal ages 1–60 (as indicated) were separated on 1% agarose gels, transferred to nitocellulose, and hybridized with CNPase (A), MBP (B), and PLP (C) ^{32}P-labeled cDNA probes. RNA extracted from telencephalon (1A), metencephalon (1D), and mesencephalon (1E) from E_{15} and E_{18} embryos were also deposited on the same gels. Exposure time of the autoradiogram was seven days except for the mesencephalon (1E) where it was 24 hours. When autoradiograms from mesencephalon were exposed for seven days, CNPase signal was clearly seen at E_{15} and MBP and PLP signals at P_1. We have chosen to present the 24-hour exposure for mesencephalon as, due to the large amount of specific messages present in this brain region, from P_9 to P_{30}, the signals after a seven-day exposure were too intense. Fig. 1F shows the autoradiograms of selected filters dehybridized and rehybridized with an α-tubulin ^{32}P-labeled cDNA probe (Ol = olfactory bulb, Ce = cerebellum, Me = mesencephalon, kb = kilobases).

et al. (1986). The in vivo study showed that this sequence of events was identical in the cerebellum than in the olfactory bulb following a caudorostral gradient. The availability of cDNA probes encoding a number of myelin proteins has provided the tools for us to investigate the developmental expression of specific transcripts coding for MBP, PLP, and CNPase in different brain areas.

Materials and Methods

Animals

Timed pregnant Wistar rats were obtained from CERJ (St.-Genest France). Embryos were taken at E_{15} and E_{18}. The first 24 hours of postnatal life was considered as postnatal day P_1. Animals were taken on P_1, P_3, P_5, P_7, P_9, P_{11}, P_{15}, P_{18}, P_{21}, P_{30}, and P_{60}. All animals were killed by decapitation and immediately processed. The dissection of the E_{15} and E_{18} embryos was performed under a binocular microscope, and from each embryonic brain the metencephalon, mesencephalon, and telencephalon were removed. The cerebellum, mesencephalon olfactory bulb, cortex, and diencephalon were separated from the brain of postnatal animals. Each brain region was immediately frozen in liquid nitrogen. Livers were taken from animals at each age as controls.

RNA Preparation

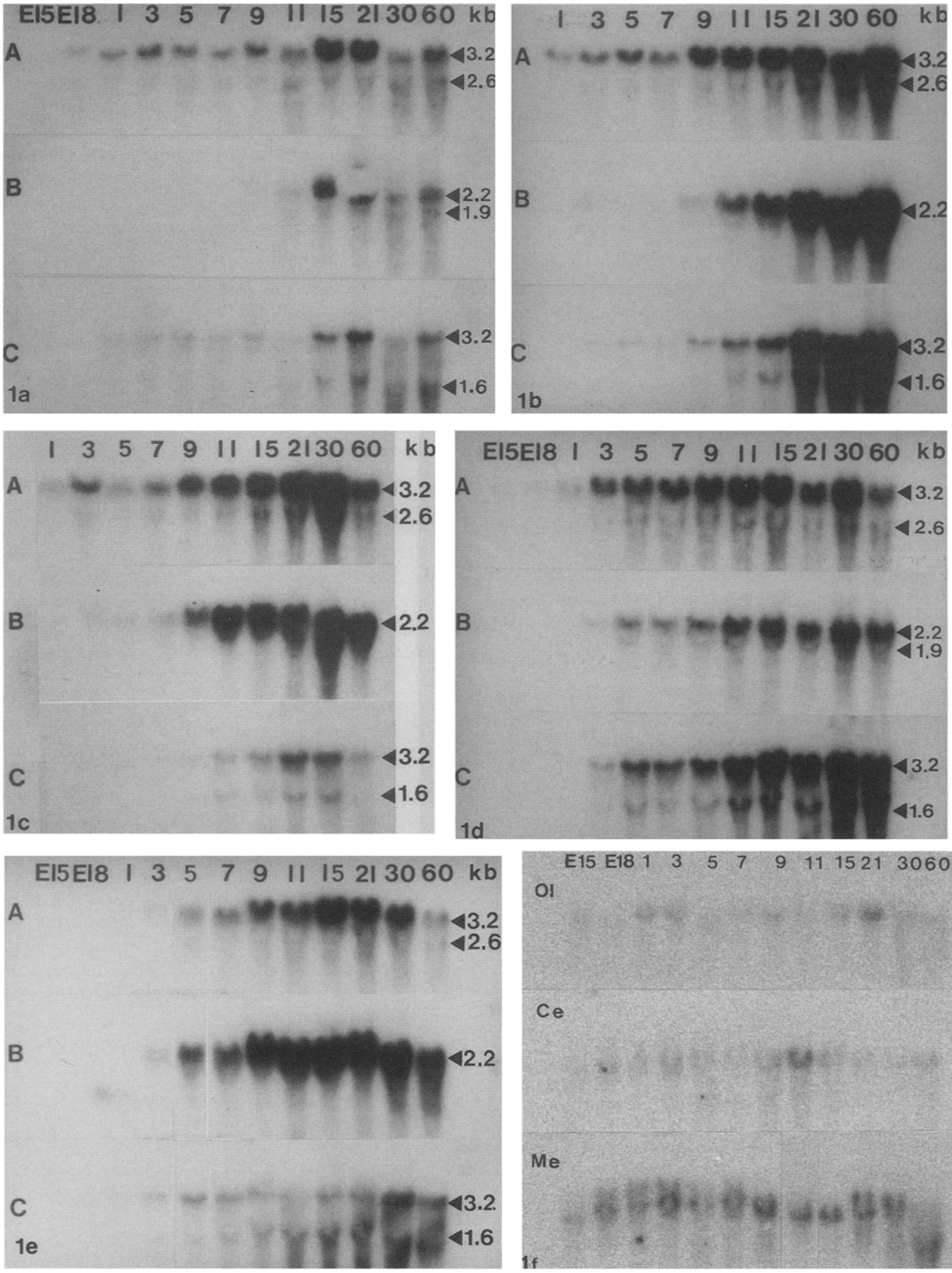
Total RNA was extracted from the frozen samples by the guanidine thiocyanate–cesium chloride method of Chirgwin et al. (1979) as modified by Civelli et al. (1982). RNA concentration was evaluated by measuring the optical density at 260 nm. For all RNA samples, the ratio of optical density at 260 and 280 nm was >1.8. In some experiments, the poly(A)$^+$RNA fraction was isolated by affinity chromatography on oligo(dT) cellulose (Aviv and Leder, 1972). The quality of the RNA preparations was routinely monitored by agarose gel electrophoresis. DNA was either absent or accounted for no more than 5–10% of the total nucleic acid.

Northern Blot Analysis

Aliquots containing 15 μg total RNA of each sample were electrophoresed in a 1% agarose gel (Seakem, Rockland, ME) in 50 mM MOPS buffer, pH 7.0, containing 2 M formaldehyde, 50 mM sodium acetate, and 5 mM EDTA and transfered to a nitrocellulose membrane (Schleicher & Schull, Dassel, FRG) as described by Thomas (1980) and modified by Faucon-Biguet et al. (1986). The nitrocellulose blots were prehybridized for 24 hours at 42°C in 50% (v/v) formamide, 10% (w/v) dextran sulfate, 5 X SSC, 50 mM sodium phosphate, pH 7.0, 1 X Denhart's solution, herring sperm DNA at 250 μg/ml, and poly(A) at 10 μg/ml. After removal of the prehybridization solution, the filters were hybridized with ^{32}P-labeled probes for 24 hours at 42°C in the same solution but without poly(A) and with herring sperm DNA at 50 μg/ml. The nitrocellulose membranes were washed twice in 2 X SSC, 0.1% SDS, twice in 0.3 X SSC, 0.1% SDS, and twice in 0.1 X SSC, 0.1% SDS. Each washing was at 65°C for 30 min. [1 X SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0; Denhardt's solution (50X) = Ficoll 5 g, polyvinylpyrrolidone 5 g, bovine serum albumin 5 g, H_2O to 500 ml]. Blots were exposed to Fuji RX films at −70°C with Cronex lighting plus intensifying screens.

Dot Blot Analysis

The experimental procedure was peformed as described by Anderson and Young (1985). Five and 10 μg of the total RNA from each sample were immobilized on nitrocellulose sheets using a BRL "Hybridot" manifold. Prehybridization, hybridization with the ^{32}P-labeled probes, and washing were performed under the same conditions as for the Northern blot analysis. After an overnight autoradiography, the dots were cut from the nitrocellulose sheets, dissolved in 1 ml NCS solubilizer (NEN) by heating at 50°C and then counted in OCS liquid scintillant (NEN, Boston, MA). Aliquots of the radiolabeled probes were directly deposited on nitrocellulose and counted in an identical manner. The


E15E18 1 3 5 7 9 11 15 21 30 60 kb
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experiments were repeated twice, and the data were similar.

cDNA Probes

The mouse MBP probe (pP18.5) used in this study has been described by Roth et al. (1986), the PLP probe (P23) was that described by Dautigny et al. (1985), and the CNPase probe (pCNP7) was that described by Bernier et al. (1987). The α-tubulin probe was a gift of P. Rataboul and J. Mallet (Gif/Yvette). Probes were radiolabeled by nick-translation (Rigby et al., 1977) with α-[^{32}P]dCTP (400 Ci/mmol, Amersham, Arlington, IL) by using a kit obtained from BRL (Gaithersburg, MD). Specific activities of $1-2 \times 10^8$ cpm/μg DNA were obtained. Lambda phage DNA restriction fragments obtained with HindIII treatment (Boehringer, Mannheim, FRG) were end-labeled with [γ-^{32}P]ATP (3000 Ci/mmol Amersham) and T4 polynucleotide kinase (Boehringer).

Results

Northern Blots

Northern blot analysis of total RNA preparations showed a characteristic pattern for each probe used (Fig. 1). CNPase gave two signals: one at 3,200 nucleotides (nt) and the other at 2,600 nt. The 3,200 nt message was the most abundant and preceded the appearance of the 2,600 nt message. The 2,600 nt signal appeared at P_1 in the olfactory bulb (Fig. 1A) and cerebral hemispheres (Fig. 1B), at P_3 in the cerebellum (Fig. 1D) and diencephalon (Fig. 1C), and at P_5 in the mesencephalon (Fig. 1E).

Two signals were also detected with the PLP probe: one of 3,200 nt and the other of 1,600 nt. The 3,200 nt message was more abundant than the 1,600 nt and, in general, both were detectable at the same date.

The MBP probe revealed a broad band ranging from 2,100 to 2,400 nt. Furthermore, in the olfactory bulb (Fig. 1A) and the cerebellum (Fig. 1D), an additional message of 1,900 nt was present. In the mesencephalon from P_{11} to P_{21}, an extra signal of 3,200 nt could also be detected on longer exposure (not shown).

Dot Blots

An example of a radioautogram of the dot blot analysis for mesencephalon is presented in Fig. 2. It is apparent that there is a progressive increase in the intensity of the signal for the probes followed by a decrease of this intensity.

Message Appearance

The earliest day of the detection of each transcript by both the Northern and the dot blot analysis showed a good concordance except for PLP in the diencephalon (Table 1). Specific mRNA coding for CNPase was at a detectable level at E_{15} in the metencephalon, mesencephalon, and telencephalon. The profile of appearance of MBP mRNA followed a caudorostral gradient. It was detected at P_1 in the mesencephalon, at P_3 in the cerebellum and diencephalon, at P_5-P_7 in the cerebral hemisphere, and at P_9-P_{11} in the olfactory bulb. This contrasts with the appearance of the PLP message, which was detected at P_1-P_3 in all five brain areas analyzed.

Developmental Profile

Depending upon the probe and the brain region examined, either a biphasic or triphasic developmental pattern was observed (Fig. 3). The biphasic profile was most obvious with the PLP probe in the olfactory bulb, the cerebral hemisphere, the diencephalon, and the cerebellum (Fig. 3A–D). It also was seen with the MBP probe in the olfactory bulb and the cerebellum (Fig. 3A,D) and with the CNPase probe in the olfactory bulb and the cerebral hemisphere (Fig. 3A,B). The maximal level of MBP and PLP messages in the cerebellum and CNPase in the cerebral hemisphere was reached at P_{30}. The triphasic pattern is most readily apparent in the mesencephalon (Fig. 3E), where each probe gave a similar profile. The initial phase consisted of a period of regular increase in the level of specific message from E_{18} to P_{11}, depending upon the particular RNA sample. During the second phase there was a dramatic increase in binding, by a factor of 4–8, from P_{11} to P_{21}, depending on the probe, followed by a sharp decline in the level of specific RNA. After this period, the level of message was relatively constant. This triphasic developmental profile was observed for the CNPase probe in the diencephalon, cerebellum, and mesencephalon (Fig. 3C–E); for the MBP probe in the cerebral hemisphere, diencephalon, and mesencephalon (Fig. 3B,C,E); and for the PLP probe in the mesencephalon (Fig. 3E). In the instances where peaks were obvious, they occurred at P_{15} for CNPase, at P_{18} for MBP, and P_{21} for PLP, regardless of the brain region.

The maximum levels of mRNA specific for each of the three probes were in the same range for a particular region. The levels were 2–4 pg/μg RNA in the olfactory bulb, 4–6 pg/μg RNA in the cerebellum, 4–8 pg/μg RNA in the cerebral hemispheres, and 25–35 pg/μg RNA in the mesencephalon. The range of levels was wider in the diencephalon, between 4 and 7 pg/μg RNA for PLP and CNPase but reaching 14 pg/μg RNA for MBP. These quantitative estimations indicate that the

mesencephalon is richest in myelin specific messages among the brain regions studied.

Controls

It appeared necessary to demonstrate that the changes in the levels of these three messages had some specificity to myelin-related events rather than being a general phenomenon that was nonspecific. For this purpose α-tubulin was chosen as a representative cellular protein. Selected filters were dehybridized and then rehybridized with an α-tubulin probe (Fig. 1F). A signal of similar intensity was seen at all ages, indicating that the developmental patterns observed with the MBP, PLP, and CNPase probes were not due to varying amounts of mRNA in the samples.

There was no hybridization signal observed with the MBP and PLP probes using liver RNA prepared from animals of all ages utilized in this study. A faint signal of 2,600 nt was seen in all samples with the CNPase probe. This latter signal can be related to the one described by Bernier et al. (1987) in the lymphoid tissue.

Discussion

Developmental profiles of MBP and PLP mRNA have already been carried out on whole brain tissue (Roach et al., 1983; Milner et al., 1985; Naismith et al., 1985; Gardinier et al., 1986; Zeller et al., 1984). In the current study the time course of appearance in different brain areas of mRNA coding specifically for MBP, CNPase, and PLP during development was examined. The appearance of specific MBP messages followed a caudorostral gradient, and this was not seen for either CNPase or PLP. The CNPase message was detectable in all the regions studied at E_{15}, but the PLP mRNA was detectable at P_1 or P_3 in the areas examined. There is existing literature discussing the appearance of myelin components in the developing brain, but there are only a few studies of regional development of myelin components with which to compare the present data.

The time course of appearance of MBP, PLP, and W_1 in the cerebellum and the olfactory bulb was studied by indirect immunofluorescence on dissociated cell preparations (Monge et al., 1986). In this latter study the date of appearance of a given antigen was chosen when one cell out of a thousand was stained by the antibody. In the present study, RNA was extracted from the whole brain region studied. Considering the differences in the methodological approaches, there is reasonable agreement between the immunocytochemical detection of MBP and the appearance of its mRNA in the cerebellum and the olfactory bulb. There is also agreement between the immunocytochemical detection of PLP and the appearance of its specific mRNA from P_1 to P_3 in the cerebellum. In the olfactory bulb, however, the PLP-specific mRNA was detectable one week prior to PLP being detectable immunohistochemically. It seems unlikely that in the olfactory bulb the PLP message would be transcribed one week before being translated. A possible explanation for this delay could be that, due to differential splicing, part of the message detected by the PLP probe might be translated into a protein that is not detected by the particular anti-PLP antibody. Perhaps this protein would be expressed between P_1 and P_7 in the olfactory bulb, and starting at P_7 the message would include PLP that is reactive with the antibody. It is difficult to compare the regional developmental appearance of CNPase mRNA and its product since the anti-W_1 antiserum previously utilized cannot be assumed equivalent to an anti-CNPase antibody (J.L. Nussbaum, personal communication). Sprinkle et al. (1978) and Miko-

Table 1. Date of appearance of the mRNAs coding for CNPase, MBP, and PLP in selected regions of embryonic or postnatal rat brain

	CNPase		MBP		PLP	
	N	D	N	D	N	D
Telencephalon	E_{15}	E_{15}				
Olfactory bulb			P_9	P_{11}	P_1	P_1
Cerebral hemisphere			P_5	P_7	P_3	P_1
Diencephalon	—	—	P_3	P_3	P_9	P_3
Metencephalon	E_{15}	E_{15}				
Cerebellum			P_3	P_3	P_3	P_1
Mesencephalon	E_{15}	E_{15}	P_1	P_1	P_1	P_1

This table compares the date of appearance as determined both by Northern (N) and dot (D) blot analysis. E = embryonic day, P = postnatal day.

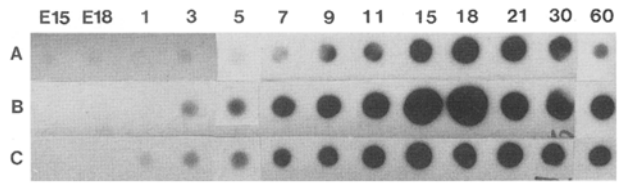

Fig. 2. Comparative dot-blot hybridization of CNPase, MBP, and PLP mRNAs in rat mesencephalon during development. Aliquots (5 μg) of total RNA extracted from rat mesencephalon of the indicated embryonic (E) or postnatal ages (days) were directly applied to nitrocellulose and hybridized with CNPase (A), MBP (B), and PLP (C) ^{32}P-labeled cDNA probes. Exposure time of the autoradiogram was 24 hours.

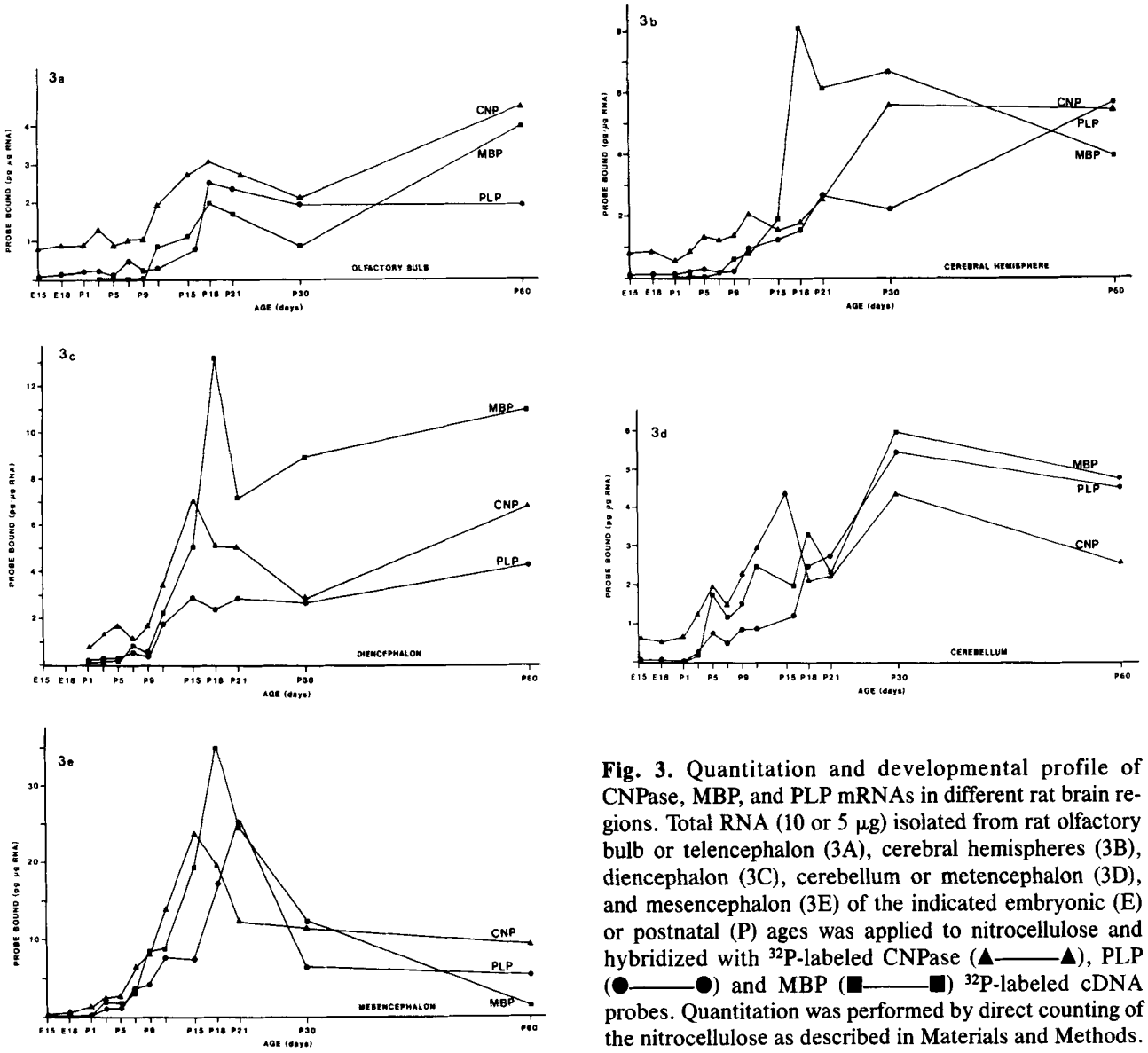

Fig. 3. Quantitation and developmental profile of CNPase, MBP, and PLP mRNAs in different rat brain regions. Total RNA (10 or 5 μg) isolated from rat olfactory bulb or telencephalon (3A), cerebral hemispheres (3B), diencephalon (3C), cerebellum or metencephalon (3D), and mesencephalon (3E) of the indicated embryonic (E) or postnatal (P) ages was applied to nitrocellulose and hybridized with ^{32}P-labeled CNPase (▲———▲), PLP (●———●) and MBP (■———■) ^{32}P-labeled cDNA probes. Quantitation was performed by direct counting of the nitrocellulose as described in Materials and Methods.

shiba et al. (1980) have reported detectable CNPase activity in all brain regions at P_4 in the rat and at P_1 in the mouse. It appears that detection of CNPase activity during embryonic life has not been examined.

In the cerebellum and olfactory bulb an additional band of 1,900 nt was observed with the MBP probe. This extra band was not observed when the poly(A)$^+$ RNA fraction was used (not shown). Similarly, in the mesencephalon an additional band at 3,200 nt present in the total RNA was observed. It is not known if these extra bands are due to a yet undescribed differential splicing of the MBP gene or if they are related to the expression of another gene cross-reacting with the MBP probe. In favor of this second hypothesis is the fact that these additional bands were region-specific.

The biphasic pattern of mRNA appearance is classical. The ascending portion probably corresponds to the proliferation and increasing number of differentiated oligodendrocytes. The plateau may be related to the steady-state level of transcription of the gene for the formation of the corresponding proteins. The slow ascending initial phase and the late plateau phase of the triphasic pattern are similar to the biphasic pattern and could have a similar interpretation. The significance of the rapid onset of the peak reactivity and its decline is less apparent. Regardless of the time of initial appearance of a specific message in any brain region, the peak for that probe occurred at the same date in all regions. Therefore, the peak was not tissue-specific but rather message-specific. It was P_{15} for CNPase, P_{18} for MBP, and P_{21} for PLP. A presumptive physiological significance of this observation would be that the temporal peak expression of transcripts

may reflect the temporal importance of each product during mylin assembly. However, this triphasic pattern does not correlate with the regional distribution of each of these proteins in the rat or the mouse during development (Delassalle et al., 1981; Mikoshiba et al., 1980; Sprinkle et al., 1978; Campagnoni and Hunkeler, 1980). Indeed, these authors have described for each of these proteins a biphasic type of developmental profile. Nevertheless, in a recent study, we reported a triphasic developmental pattern for the activity of another key enzyme of myelinogenesis, the UDP galactose: ceramide galactosyltransferase (Monge et al., 1988).

The sharp increase and decline of a message, especially in the mesencephalon, over a brief period of time seems impressive. It appears unlikely that this is the result of an accelerated increase in the rat of either oligodendroglial cells proliferation or the cells differentiation. These cell numbers are believed to increase linearly with time and have achieved their steady-state levels by P_{10}–P_{14}. Therefore, the accelerated increase in the level of specific messages occurs at a time when there is little increase in number or maturation of the oligodendrocytes. A triphasic pattern has been observed for α-cardiac actin (Bains et al., 1984) and skeletal myosin heavy chain (Weydert et al., 1987) mRNA. As we have measured only the amount of specific transcripts coding for MBP, PLP, and CNPase, we do not know if the peak of message observed is due to an increased rate of gene transcription, a developmentally regulated enhancer, or alterations of processing or turnover of the corresponding mRNA. The nature of the signal(s) responsible for increased mRNA related to MBP, CNPase, and PLP is unknown. The separate peaks occur at P_{15}, P_{18}, and P_{21}. This postnatal period corresponds to the developmental stage in the rat when myelination is the most intense. The observation that the peak of mRNA is probe- but not region-dependent suggests a common signal, perhaps neuronal. This signal might occur simultaneously in the whole brain, regardless of the caudorostral gradient of myelination (Yakovlev and Lecours, 1967). Dot blot analyses of cytoplasmic RNA from primary cultures of rat brain oligodendrocytes have been carried out with MBP and PLP cDNA probes (Macklin et al., 1986). These studies reported elevated levels of transcripts corresponding to these probes when the oligodendrocytes were cocultured with embryonic chick spinal cord neurons. This report, together with the present in vivo study, strengthens the hypothesis that a neuronal signal is responsible for the increased level of myelin related mRNA.

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