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Thyroid hormone affects Schwann cell and oligodendrocyte gene expression at the glial transition zone of the VIIIth nerve prior to cochlea function

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SUMMARY

All cranial nerves, as well as the VIIIth nerve which invades the cochlea, have a proximal end in which myelin is formed by Schwann cells and a distal end which is surrounded by oligodendrocytes. The question which arises in this context is whether peripheral and central parts of these nerves myelinate simultaneously or subsequently and whether the myelination of either of the parts occurs simultaneously at the onset of the cochlea function and under the control of neuronal activity. In the present paper, we examined the relative time course of the myelinogenesis of the distal part of the VIIIth nerve by analyzing the expression of peripheral protein P0, proteolipid protein and myelin basic protein. To our surprise, we observed that the expression of myelin markers in the peripheral and central part of the intradural part of the VIIIth nerve started simultaneously, from postnatal day 2 onwards, long before the onset of cochlea function. The expression rapidly achieved saturation levels on the approach to postnatal day 12, the day on which the cochlea function commenced. Because of its importance for the neuronal and morphological maturation of the cochlea during this time, an additional role of thyroid hormone in cochlear myelinogenesis was considered. Indeed, it transpires that this hormone ensures the rapid accomplishment of glial gene expression, not only in the central but also in the peripheral part of the cochlea. Furthermore, an analysis of the thyroid hormone receptors, TRα and TRβ, indicates that TRβ is necessary for myelinogenesis of the VIIIth nerve. Rapid thyroid hormone-dependent saturation of myelin marker gene expression in Schwann cells and oligodendrocytes of the VIIIth nerve may guarantee nerve conduction and synchronized impulse transmission at the onset of hearing. The thyroid hormone-dependent commencement of nerve conduction is discussed in connection with the patterning refinement of central auditory pathways and the acquisition of deafness.

Key words: Thyroid hormone, Thyroid hormone receptor α, Thyroid hormone receptor β, Schwann cells, Peripheral protein zero, Myelin basic protein, proteolipid protein, Rat

INTRODUCTION

Myelination of central pathways has been traditionally regarded as a marker for the onset of function. This is commensurate with the observation that the process of myelinization via peripheral Schwann cells (Scherer et al., 1992), as well as central oligodendrocytes (Demerens et al., 1996), is dependent on or influenced by neuronal activity.

In the auditory system, observations relating to the course of myelination in the human brainstem support the progressive myelinization of central auditory pathways beyond the onset of the auditory function (Langworthy, 1933; Moore et al., 1995). Although the temporal differences between myelinization of the intradural course of the VIIIth nerve, the brainstem and the inferior colliculus (Moore et al., 1995) are minor, the myelinization of auditory pathways nevertheless progresses in a centripetal fashion (Pujol, 1972). In the auditory system, the acousticomotor reflex and evoked auditory brainstem responses, both of which need rapid and synchronized activation of neuronal pathways, can be detected at the onset of hearing. Synchronized conduction necessarily depends on myelinogenesis and therefore a tight correlation between the time of myelination of the proximal part of the VIIIth nerve and the time of hearing onset is essential for the normal development of hearing (Moore et al., 1995). In contrast to the view which claims that myelinization starts at the onset of the auditory function, myelin sheaths were noted in axons within the cochlea before the onset of recordable action potentials (Pujol and Hilding, 1973; Romand et al., 1976; Rubel, 1978). Thyroid hormone has been shown to influence neuronal and morphogenetic processes before the cochlea is functional (Uziel et al., 1983a,b, 1985). In order to improve our understanding of the relative time course of peripheral and central myelination in sensory systems in

general, and the auditory system in particular, we studied the spatiotemporal distribution of the myelin marker mRNA in the VIIIth nerve at the glial transition zone in oligodendrocytes and Schwann cells, in relation to the onset of hearing and also a presumed effect of thyroid hormone. In the peripheral nervous system the peripheral protein zero (P0) and the myelin basic protein (MBP) were used as markers for myelin and in the central nervous system proteolipid protein (PLP) as well as MBP were chosen (Schaeren-Wiemers and Gerfin-Moser, 1993; for a review see Lees and Brostoff, 1984; Lemke, 1988, 1995). The time course for the appearance of mRNA of these markers, has been shown to largely correspond to that of myelination (Campagnoni and Hunkeler, 1980). We were surprised to note that both peripheral and central myelin markers appeared in the intradural part of the cochlea much earlier than the onset of cochlea function, simultaneous to and dependent on the rise of the plasma thyroid hormone level. Furthermore, the expression pattern had a profile comparable to that of thyroid hormone receptor, TRβ. One role of thyroid hormone (TH), namely to guarantee the developmental link to the schedule of central auditory processes is discussed in the context of the acquisition of deafness under hypothyroid conditions.

MATERIALS AND METHODS

Animals and drug administration

Wistar rats were obtained from Interfauna (Tuttlingen, Germany). To induce hypothyroidism, methyl-mercapto-imidazol (MMI; 0.02%) was administered in the drinking water of the dams from the indicated embryonal day after conception (usual E17) and was continued until the pups were killed and analyzed. Hypothyroidism was controlled by determination of plasma thyroxine (T4) and triiodothyronine (T3) levels of individual pups of the litter (see below). The effect of hypothyroidism on auditory function was controlled via analysis of auditory evoked brainstem responses (ABR) as described by Knipper et al. (1998). Hypothyroid pups remained with their dams until the day of decapitation.

The care and use of the animals during the course of this study were in accordance with the guidelines of the Declaration of Helsinki.

T3/T4 determination

For the quantitative determination of thyroxine (T4) or triiodothyroinine (3,5,3′-L-triiodothyronine; T3) levels in rat serum, the CIBA-Corning automated chemiluminescent system (ACS-T4, ACS-T3) and the ACS 180 chemiluminometer were used. Blood was collected when animals were killed for the examination of the cochlea.

Tissue preparation

Sprague-Dawley rat pups of postnatal ages P1 to P28 were used for this study. The day of birth was taken as P0. Under anesthesia, the cochleae were rapidly removed, fixed by immersion for 2 hours in 2% paraformaldehyde and cryosectioned as described by Knipper et al. (1995a, 1996, 1997). For northern blotting, cochleae, tissue dissected from the brainstem, the area of the inferior colliculus and the area of the auditory cortex around the temporal lobe were immediately frozen in liquid nitrogen and stored at −80°C before use.

Immunocytochemical staining

Sections were thawed, preblocked and stained with antibodies as described by Knipper et al. (1995a, 1996, 1997). Polyclonal antisera against myelin binding proteins MBP and PLP came from Biotrend (Köln, Germany) and were diluted 1:600 MBP and 1:50 PLP. Polyclonal (rabbit) anti-thyroid hormone receptor TRα1 (TRα1-403, aa403-410), TRα2 (TRα2-431; aa431-451), polyclonal (rabbit) anti-thyroid hormone receptor TRβ1 (TRβ-62; aa62-82), and monoclonal (mouse) anti-thyroid hormone receptor TRβ1 (MA1-216) were obtained from Dianova (Hamburg, Germany) and diluted 1:50. These antibodies are specific for the individual receptors, immunoprecipitate the appropriate receptor proteins and cross react with human as well as rat tissue (Macchia et al., 1990; Lin et al., 1991; Falcone et al., 1992). Polyclonal antibody to TRβ2 was prepared as described below. Sections were viewed and photographed using an Olympus AX70 microscope equipped with epifluorescence illumination. Photomicrographs were taken on Kodak TMY-400 film rated at 400 ASA.

Detection of TR proteins, MBP and PLP upon western blotting

The organ of Corti was collected from postnatal rats of indicated age. The equivalent of 5 organ of Corti (5 μg protein) were separated on gradient acrylamide gels and protein transfer was performed as described by Knipper et al. (1995a). PVDF membranes were incubated with 5% dry milk powder / PBS (pH 7.4) and then incubated with an anti-MBP (1:3000), anti-PLP (1:3000) (Biotrend, Köln, Germany) or with thyroid hormone receptors (Dianova, Hamburg, Germany). Thyroid hormone receptor TRβ1 antibody (Clone J 52) was diluted 1:1500 and polyclonal rabbit thyroid hormone receptor TRβ1 antiserum (PA1-213), polyclonal rabbit thyroid hormone receptor TRα1 antiserum (PA1-211) and polyclonal thyroid hormone receptor TRα2 antiserum (PA1-212) were used at a 1:1000 dilution. TRβ2 antiserum was prepared as described below and was used at a 1:1500 dilution. Signals were visualized with the Enhanced Chemiluminescence Detection System (ECL) according to the manufacturer's instructions (DuPont, NEN, Boston, MA, USA).

TRβ2 serum preparation and characterization

An antiserum to TRβ2 was prepared coupling peptides corresponding to amino acids 131-145 of rat TRβ2 (Hodin et al., 1989) with KLH-MBS (Eurogentec, Ougree, Belgium). The TRβ2-specific sequence is not present in other known TRs and a search of the National Biomedical Research Foundation protein database (Lechan et al., 1993) did not find any comparable amino acid sequences. The coupled peptides were injected into two New Zealand white rabbits (Eurogentec, Ougree, Belgium). The synthetic TRβ2 [131-145] peptide blocked immunoprecipitation of TRβ2. Similarly, immunocytochemical controls showed marked reduction or complete abolition of immunostaining of TRβ2 in all regions of the brain studied after preadsorption of the TRβ2 antiserum with TRβ2 peptide. Also there was no specific nuclear immunostaining when the primary antiserum was substituted for preimmune serum.

Isolation of myelin

Myelin from the peripheral nervous system was isolated from the sciatic nerve of rats at P16, because cochleae dispose of Schwann cells as well as oligodendrocytes during that time. Myelin from the central nervous system was isolated from the brainstem, the VIIIth nerve or spinal cord of rats at P16. Myelin was prepared according to the protocol of Colman et al. (1982) as described by Caroni and Schwab (1988) and Rubin et al. (1995). In brief, tissue (10 g/40 ml) was homogenized in 0.25% sucrose, 20 mM Hepes (pH 7.4), 5 mM EDTA, 5 mM iodacetamid, 2 μg/ml aprotinin, 1 mM PMSF, 10 μM leupeptin, 10 μM pepstatin with polydron 3× 30 seconds. 10 ml fractions diluted 1:1 in 2 M sucrose, 20 mM Hepes were layered over 3 ml of 2 M sucrose, 20 mM Hepes. 3 ml of 0.85 M sucrose, 20 mM Hepes (pH 7.4) and 2 ml of 0.85 M sucrose were layered on top. The gradient was centrifuged in SW 28 at 70,000 ***g*** for 4 hours at 4°C. The floating myelin layer was removed and separated on gradient acrylamide gels.

Probe transcription

MBP cDNA, cloned in pBR322 as described by Roach et al. (1983) and PLP, cloned in pUC18 as described by Milner et al. (1985) was subcloned as described by Schaeren-Wiemers and Gerfin-Moser (1993) and Lemke and Axel (1985). Subcloned probes were kindly provided by M. Frank and M. E. Schwab, University Zurich, Switzerland; P0 cDNA, subcloned in pBS KS were kindly provided by G. Lemke, Salk Institute San Diego, CA, USA. The full-length cDNA clone of rat TRβ1 subcloned into Bluescript vector pBS KS^+ was kindly given by R. J. Koenig, University of Michigan, Ann Arbor, USA. TRα1 and TRα2 cDNA subcloned in CMV-vector containing the polylinker region from pBS KS+ were kindly provided by M.A. Lazar, University of Pennsylvania, USA. The digoxigenin-labeled RNA probes were transcribed either from T7 or T3 for antisense, or sense promoter sites in PBS in the presence of digoxiginin-UTP (Boehringer, Mannheim, Germany). Digoxigenin-labeled actin RNA-probe was obtained from Boehringer Mannheim, Germany, ready for use and was diluted 1:5000. For in situ hybridization the full-length transcripts were cut to an average length of 200 base pairs by alkaline hydrolysis (Cox et al., 1984), precipitated in ethanol and resuspended in hybridization buffer (Amersham, Braunschweig, Germany, RPN3310) in 50% formamide.

In situ hybridization of digoxigenin-labeled RNA probes

Fixation, embedding and sectioning

Cochleae were dissected, fixed and sectioned as described for immunohistochemistry.

Probe hybridization

In situ hybridization according to Strotmann et al. (1995) was modified as follows. Fixed sections were dried and riboprobes were applied for overnight hybridization in a moist chamber at 60°C. The sections were washed twice in 0.1× SSC (300 mM sodium chloride, 30 mM sodium citrate, pH 7.0) at 60°C for 30 minutes. After a brief wash in buffer A (0.1 M Tris-HCl, 0.15 M sodium chloride, pH 7.5), sections were blocked in buffer A containing 0.5% blocking reagent (Boehringer Mannheim, Germany, 1096176) and exposed to anti-digoxigenin antibody conjugated to alkaline phosphatase (Fab fragments, Boehringer Mannheim, Germany). Exposure of color solution using nitroblue tetrazolium salt (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) was extended for up to 20 hours. Sections were photographed under Nomarski optic using Olympus AX70 microscope.

Northern blot analysis

The cartilaginous or bony capsule was carefully dissected from the cochlea of postnatal rats at the indicated age. The opened spiral canals inclusive of the modiolus around which they wind, were collected, immediately frozen in liquid nitrogen and stored at −70°C until needed. Alternatively, brainstem, inferior colliculus and auditory cortex at the level of the temporal lobe were dissected and immediately frozen in liquid nitrogen and stored at −70°C until needed.

Detection of mRNA was performed with the DIG System as recently described (Rueger et al., 1996). For verification of similar level of mRNA the actin RNA probe was used. As actin (Poddar et al., 1996) as well as G3PDH (Freerksen et al., 1984) have both been shown to be altered under the influence of TH, the effect of TH on mRNA levels was examined using mRNA isolated from a similar number of cochleae. Only when identical results were obtained in three independent experiments were these data used for quantification analysis.

Northern blot were analyzed using a LKB UltrascanII Enhanced Laser Densitometer. The image analyzer was calibrated in O.D. units and relative O.D. were determined as average O.D. from three independent experiments.

RESULTS

The distribution of P0, MBP and PLP mRNA in the cochlea at P10 clearly demonstrated the exclusiveness of the distinct probes for hybridization with either myelin proteins of peripheral and / or central myelin, respectively (Fig. 1). P0 and MBP probes cross react with the distal part of the $VIII^{th}$ nerve, the Nervus cochlearis, which is surrounded by Schwann cells and bifurcates from the peripheral and inferior portions of the nerve trunk to more basal cochlea turns and from the central regions towards more apical cochlea turns (Fig. 1, P0, MBP). The glial transition zone from Schwann cells to oligodendrocytes is localized at the center of the modiolus where the Nervus cochlearis and Nervus vestibularis join to form the Nervus vestibulo-cochlearis ($VIII^{th}$ nerve) which in turn enters the brainstem via a short intradural course. Using neighboring sections of the same cochlea, it became evident that in this part of the modiolus no P0 mRNA were detected (Fig. 1, P0, arrow), while both MBP probes and PLP probes exhibited strong hybridization signals at the central part of $VIII^{th}$ nerve where oligodendrocytes wrap the nerve trunk (Fig. 1; MBP, PLP, arrow CNS).

The onset of myelin marker expression occurred as a gradient along the tonotopic axis of the cochlea. MBP mRNA was first detected in the basal cochlea turn at about P2 and in the apical turn at about P6, followed about 2 days later by P0 mRNA (data not shown). The day on which PLP expression was detected at the center of the modiolus varied between P6 and P8/P9 depending of the section level.

When TH plasma levels were depleted prior to embryonal day 17 (E17), the expression of myelin markers by both Schwann cells and oligodendrocytes surrounding the $VIII^{th}$ nerve was significantly retarded. Fig. 2 shows the distribution of P0 (Fig. 2A) and MBP (Fig. 2B) in the basal turn of the cochlea at P4, P8 and P12 in euthyroid animals (control) and hypothyroid animals (hypo). In controls, increasing amounts of P0 mRNA (Fig. 2A) and MBP mRNA (Fig. 2B) could be detected with increasing age at P4, P8 and P12 along the fibers that pass from the spiral ganglion neurons (SG) in the Rosenthal's canal into the osseous spiral lamina (OSL) then project towards the hair cells of the organ of Corti (Fig. 2A,B). In the absence of TH, neither P0 mRNA nor MBP mRNA were observed at P4 (Fig. 2A,B, hypo, P4) and low levels of mRNA were observed later (Fig. 2A,B, hypo). As with P0 and MBP expression in Schwann cells, MBP and PLP expression in oligodendrocytes that wrap the more central intradural part of the $VIII^{th}$ nerve, was significantly retarded in the absence of TH (Fig. 3). While in controls, MBP mRNA and PLP mRNA were first noted at the glial transition zone within the modiolus at P6/P8, in the absence of TH, even at P11 MBP and PLP hybridization signals were still significantly reduced in comparison to the controls (Fig. 3).

Semi-quantification of myelin marker mRNA using the northern blot approach confirmed the significant effect of TH on Schwann cell and oligodendrocyte gene expression in the cochlea during the first 12 postnatal days. As shown in Fig. 4, P0 is encoded in the same single mRNA species of an expected 1.9 kb at all developmental stages. In the absence of TH, the developmental rise of mRNA levels was significantly retarded. The mRNA levels in three experiments were quantified using UltrascanXL Enhanced Laser Densitometry, averaged and

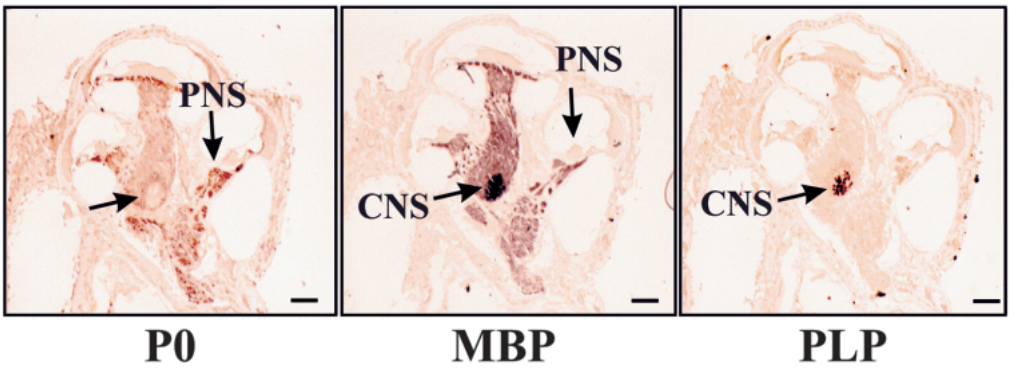

Fig. 1. Distribution of P0, MBP and PLP mRNA in rat cochlea at P10. Myelin markers were detected in neighboring cryosections using in situ hybridization and DIG-labeled riboprobes as described under methods. P0 mRNA was detected in Schwann cells ensheathing peripheral nerves (PNS) which project to the organ of Corti. MBP mRNA recognized both the central part of the VIIIth nerve ensheathed by oligodendrocytes (CNS) as well as Schwann cells (PNS), while PLP mRNA was only detected in oligodendrocytes (CNS). Bar, 200 μm.

compared with the rise of the triiodothyronine (T3) plasma level within the same time limit (Fig. 4B). P0 mRNA was first detected in the cochlea at about P3 and the level increased approximately 10-fold to a peak around P10 to P12. It then declined towards a steady level when nearing P28 (Fig. 4B, control). The slope of the rise was very similar to the rise of T3 during the first postnatal days (Fig. 4B, dotted line). Under hypothyroid conditions, however, P0 expression reached the

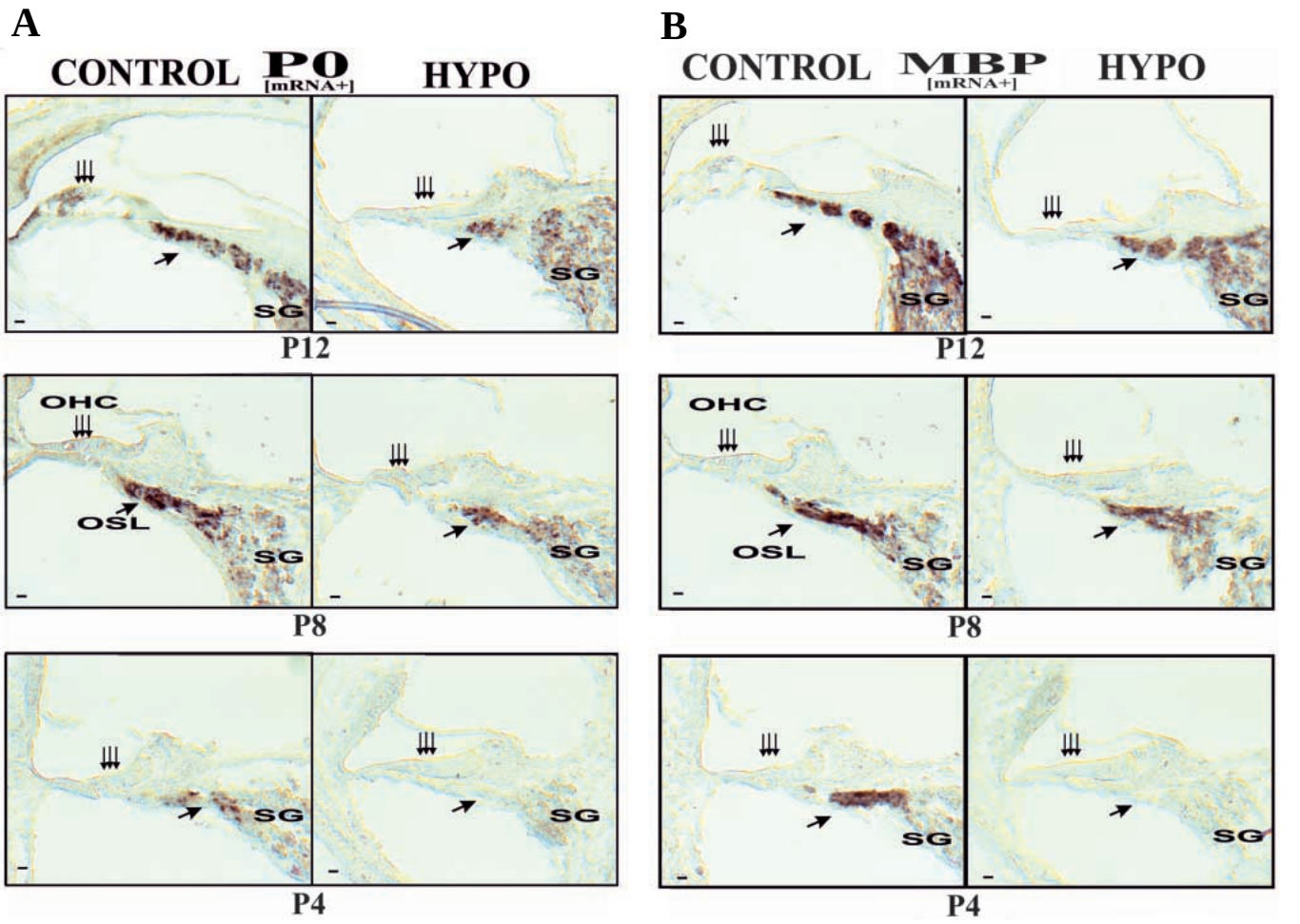

Fig. 2. Distribution of P0 mRNA (A) and MBP mRNA (B) in Schwann cells surrounding fibers in the osseous spiral lamina of the basal cochlea turn of untreated (control) and hypothyroid (hypo) animals at the indicated postnatal ages detected by in situ hybridization. Note the consistently weaker expression of P0 and MBP under hypothyroid conditions. OHC and triple arrows, outer hair cells; SG, spiral ganglia cells; OSL and arrow, osseus spiral lamina. Bar, 10 μm.

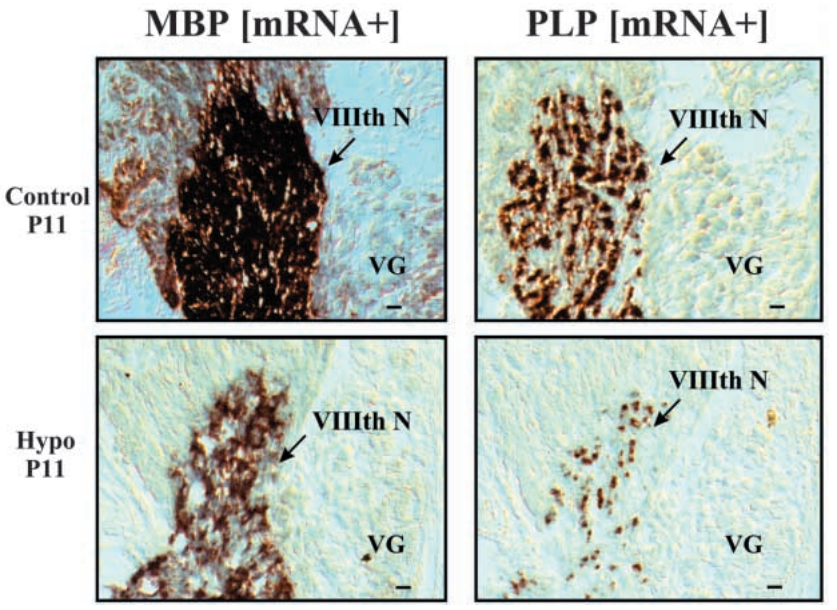

Fig. 3. MBP and PLP mRNA in the central part of the VIIIth nerve in untreated (control) and hypothyroid (hypo) animals at P11. Glial transition zone between Schwann cells and oligodendrocytes is always localized at the level of the vestibular ganglion cells. Note the significantly lower level of MBP and PLP mRNAs under hypothyroid conditions. VG, vestibular ganglion. Bar, 10 μm.

steady state level of adults at P28, never accomplishing the developmental peak of P10/P12 (Fig. 4B; hypo).

The MBP probe recognized a mRNA of the expected size of 2.1 kb at all developmental stages examined. It was detected significantly later in the absence of TH (Fig. 5A). Semi-quantification demonstrates that in controls, the expression of MBP achieved saturating levels during the first 12 postnatal days (Fig. 5B, control), comparable to the rise of T3 plasma levels, while in the absence of TH similar levels of mRNA were only accomplished when nearing P28 (Fig. 5B, hypo).

PLP probes detected both known mRNAs of the PLP gene in the developing cochlea; the more abundant 3.2 kb mRNA and the less abundant 1.6 kb mRNA (Fig. 6A). In probable agreement with differences in the abundance of the distinct mRNA, the 3.2 kb PLP mRNA was detected in higher amounts than the 1.6 kb PLP mRNA, which explains the earlier detection of the 3.2 kb mRNA (Fig. 6A). Semi-quantification of three independent experiments revealed the 3.2 kb PLP mRNA as early as P2 (Fig. 6B, control) at the same time as MPB and P0 mRNA were initially detected in the cochlea. As with MBP and P0, PLP mRNA also accomplished saturating levels in controls at about P12 (Fig. 6B, control), while in the absence of TH, PLP mRNA levels eventually achieved control levels at the end of the fourth postnatal week (Fig. 6B, hypo). Once again, mRNA levels rose in a similar fashion to the gradient of the T3 plasma level (Fig. 6B, dotted line).

The retardation of the glial gene expression at the periphery of the auditory system under hypothyroid conditions did not induce any permanent hypoexpression of myelin markers in higher central auditory brain regions, as revealed by similar MBP and PLP mRNA levels at P28 in the brainstem, inferior colliculus and auditory cortex. The northern blot approach showed similar results in the midbrain and cerebral cortex tissue (data not shown). It became evident that, as in the cochlea, TH mostly affects PLP and MBP mRNA expression in higher auditory brain centers during the first 2 postnatal weeks, with more long lasting effects of hypothyroidism in cortical than in brainstem regions (data not shown).

So far, TH has been shown to affect gene expression in oligodendrocytes, but not in Schwann cells, the effect of which has been specified by the involvement of thyroid hormone receptors. To further analyze the role of TH,

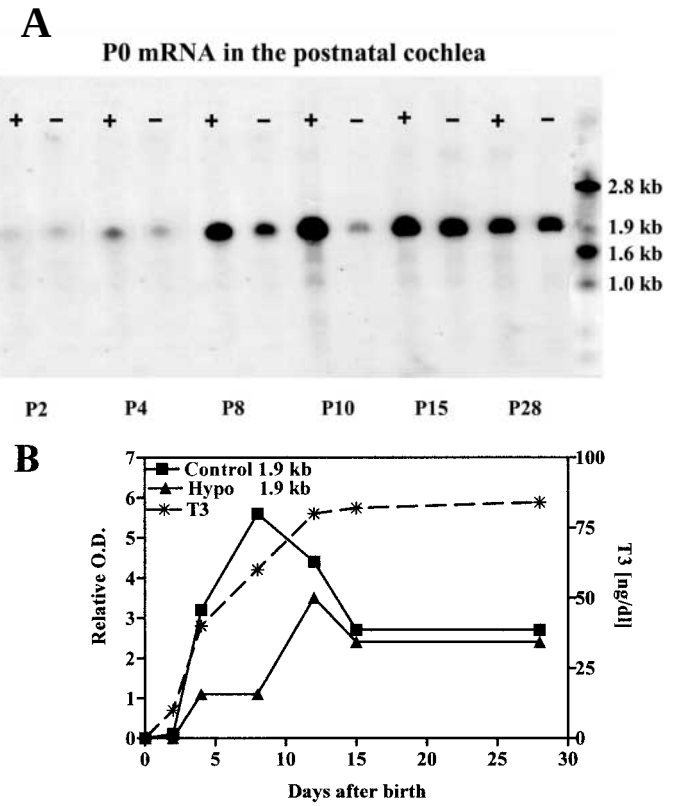

Fig. 4. Effect of neonatal hypothyroidism on P0 mRNA levels in cochleae of rats. (A) Northern blot of untreated (+) and hypothyroid (−) rats of the ages indicated. P0 probes hybridize with a single mRNA of an expected size of 1.9 kb. (B) The slope of the expression profile of P0 in untreated (control) and hypothyroid animals (hypo) is compared to the slope of triiodothyronine T3 plasma level (dotted line). Relative O.D. indicates average O.D. of three independent experiments.

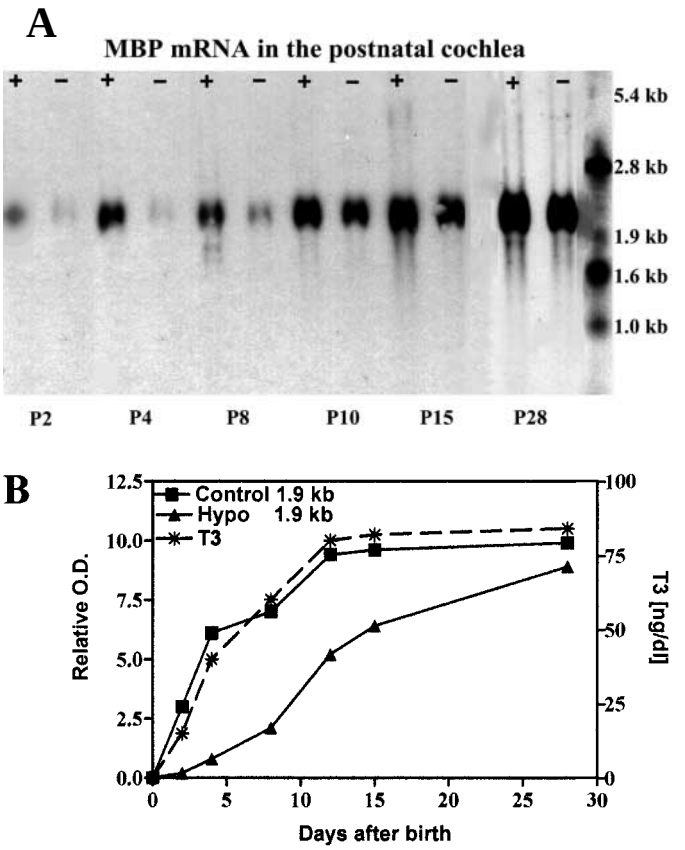

Fig. 5. Effect of neonatal hypothyroidism on MBP mRNA levels in cochleae of rats. (A) Northern blots of untreated (+) and hypothyroid (–) rats of the indicated age. MBP probes hybridize with a single mRNA of an expected size of 2.1 kb. (B) The slope of the expression profile of MBP in untreated (control) and hypothyroid animals (hypo) is compared to the slope of triiodothyronine T3 plasma level (dotted line). Relative O.D. indicates average O.D. of three independent experiments.

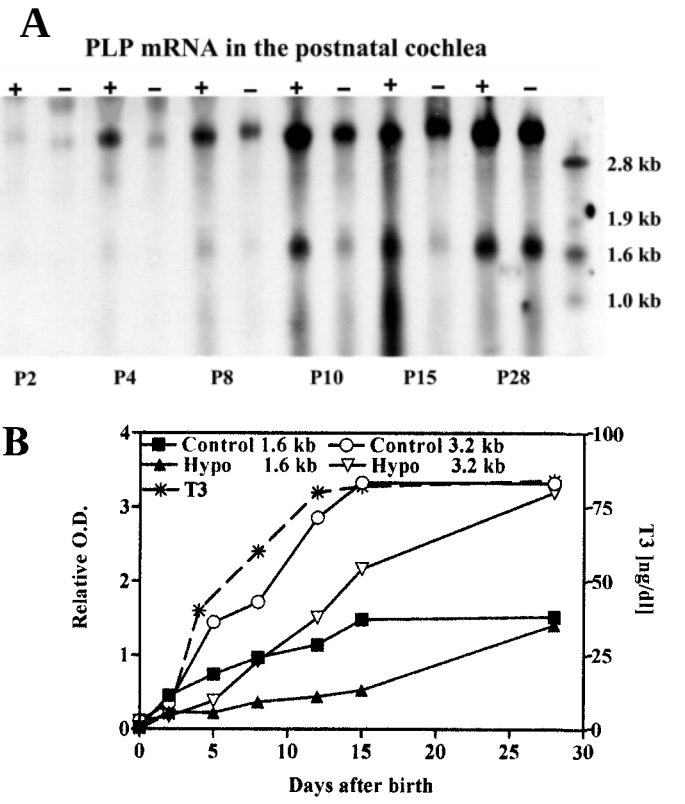

Fig. 6. Effect of neonatal hypothyroidism on PLP mRNA levels in cochleae of rats. (A) Northern blot of untreated (+) and hypothyroid (–) rats of the indicated ages. PLP probes cross hybridize with expected 3.2 kb and 1.6 kb mRNA. (B) The slope of the expression profile of myelin markers of control and hypothyroid animals is compared to the slope of triiodothyronine T3 plasma level (dotted line). Relative O.D. indicates average O.D. of three independent experiments.

particularly in Schwann cell gene expression, we examined the expression of thyroid hormone receptors in isolated myelin fractions of both the peripheral (PNS; sciatic nerve) and central nervous system (CNS; brainstem). Using mono and polyclonal antibodies, we observed the distinct expression of TRα1, TRα2 and TRβ2 in the myelin of the CNS, while TRα1 and TRβ1, but not TRα2 or TRβ2 were found to be expressed in myelin fractions of the PNS (Fig. 7). For the first time this indicates the distinct expression of thyroid hormone receptors in Schwann cells and their presumptive differential expression in glial cells of the PNS and CNS. The distinct nature of peripheral and central myelin fractions was underlined when we analyzed the expression of PLP and MBP in both myelin fractions (Fig. 7). PLP proteins of the appropriate size of 35 kDa were found in myelin fractions from the CNS but not PNS and, as expected, MBP proteins varying between 21 kDa and 14 kDa were found in higher amounts in myelin fractions from the CNS than from the PNS (Fig. 7).

When we attempted to visualize TR receptor proteins in Schwann cells within the cochlea, a presumptive expression of TR in glial cells could not be distinguished from that in spiral ganglia cells (data not shown). In a first approach we therefore focused on TR expression in the central intradural course of the VIIIth nerve. Here, as determined by the western blot approach, TRβ2 proteins (Fig. 8), but not TRβ1 (not shown) were localized in the central intradural part of the VIIIth nerve in a similar distribution pattern to the MBP and PLP mRNAs (Fig. 8).

In case TH may effect the acceleration of myelin markers on the distinct action of thyroid hormone receptors, the variation of TR expression during the postnatal development of the cochlea may indicate distinctive roles of TR subtypes. We therefore analyzed the alteration of TRs in the cochlea during postnatal development using the northern blot approach (Fig. 9). The TRα1 probe only recognized the 5.0 kb mRNA as expected, while the TRα2 probes cross-hybridized with the 5.0 kb mRNA (TRα1) and the 2.6 kb mRNA (TRα2) (Fig. 9A). As a reverse transcribed full-length clone, TRβ1 riboprobes cross-hybridized with both TRβ1 and TRβ2 mRNA, both of which are about 6.0 kb (Fig. 9B). While TRα receptor mRNA levels showed no significant alteration on P2, P8 or P13, TRβ mRNA were raised in the cochlea from P2 onwards, peaked at about P8 and slightly declined towards a lower consistent level. We used an actin

riboprobe of 1.8 kb to ensure the use of an approximately similar mRNA content. Semi-quantification of TRα1/2 mRNA levels revealed no correlation with the variation of the T3 plasma level (Fig. 9A, lower panel), while a significant rise in TRβ mRNA from P1/P2 onwards occurred in a similar fashion to the gradient of the T3 plasma level (Fig. 9B, lower panel).

DISCUSSION

In the present study, we show that the expression of myelin markers, P0, MBP and PLP in glial cells surrounding the peripheral and proximal part of the VIIIth nerve, start simultaneously and, under the control of thyroid hormone, long before cochlear activity. At least for the VIIIth nerve, the question concerning the time course of myelination of peripheral and central parts of the nerve in relation to the onset of cochlea function and the beginning of the myelination of more central brain areas (Moore et al., 1995) is given a strikingly simple answer: the early rise of thyroid hormone plasma levels at the beginning of thyroid gland function controls an acceleration of myelin protein expression in the peripheral as well as central part of the VIIIth nerve. This, then guarantees saturating levels of myelin genes in the distal part of the cochlear nerve when neuronal and morphogenetic maturation of the cochlea initially permits transduction of sound signals.

The finding of significant acceleration of P0, MBP and PLP expression in Schwann cells and oligodendrocytes in the peripheral and central auditory pathway under the control of TH, indicates that myelin gene expression starts in Schwann cells and oligodendrocytes in the intradural part of the VIIIth nerve. From there, it spreads in a peripheral and central direction, accomplishing saturating levels within the first two postnatal weeks. The time course for TH-dependent saturation of the myelin marker mRNA level is similar to that recently found in other central brain areas (Rodriguez-Pena et al., 1993; see for a review Oppenheim et al., 1994; Bernal and Nunez, 1995). Myelination of central and peripheral nerves has traditionally been regarded as a marker for the onset of function establishing significant dependency on neuronal activity (Scherer et al., 1992; Scherer, 1997; Demerens et al., 1996). Thus, a two step model for myelinogenesis may be considered: a TH-dependent acceleration of glial gene expression precedes an activity-dependent step, which may for example include the continued increase in protein expression and wrapping of myelin sheets. As such, the accomplishment of myelin gene expression in the peripheral and central part of the auditory pathway during the first 12 postnatal days, may be the prerequisite for nerve conduction and spreading of action potentials from the periphery to the brainstem. This process occurs at the same time as cellular and morphological maturation of the cochlea initial permits sound signal transduction (Rubel, 1978). In keeping with this, the myelinogenesis process of the central auditory system has been described as progressing from the onset of cochlea function onwards (Langworthy, 1933; Moore et al., 1995) causing a gradual improvement of hearing thresholds (Moore et al., 1995). Moreover, acousticomotor reflex as well as evoked auditory brainstem responses, both of which need rapid, synchronized conduction of neuronal pathways, can be observed from P12 onwards (Moore et al., 1995). Such a two-step myelination model may also be an explanation for irreversible hypomyelination, caused by hypothyroidism (Legrand, 1980; Noguchi et al., 1982). However, spontaneous normalization of myelin gene expression even in the absence of TH is documented. In this situation, permanent hypomyelination may simply be due to a retarded start in conduction and impulse transmission, the well-timed onset of which is required for the second activity-dependent step in the myelination process.

The present paper demonstrates that TH affects gene expression in oligodendrocytes as well as in Schwann cells. While the effects of thyroid hormone on myelinogenesis in the central nervous system have been shown in manifold studies (Farsetti et al., 1992; Tosic et al., 1992; Rodriguez-Pena et al., 1993; see for a review Dussault and Ruel, 1987; Oppenheim et al., 1994; Bernal and Nunez, 1995), so far there exists no direct evidence that thyroid hormone controls Schwann cell gene expression. In agreement with the present finding, however, previous studies report a transient expression of TRs in Schwann cells during the postnatal period in rats (Barakat-Walter, 1993; Glauser et al., 1997). While their role during development has so far remained unclear, an injury-induced upregulation of TRs in peripheral glial cells has been discussed in relation to the presumptive role they play in nerve regeneration (Walter, 1993; Walter and Droz, 1995). Progesterone is the only hormone which has been described as having an influence on Schwann cell differentiation (Koenig et al., 1995). The acceleration of Schwann cell gene expression by TH, demonstrated in the present study, suggests that it may

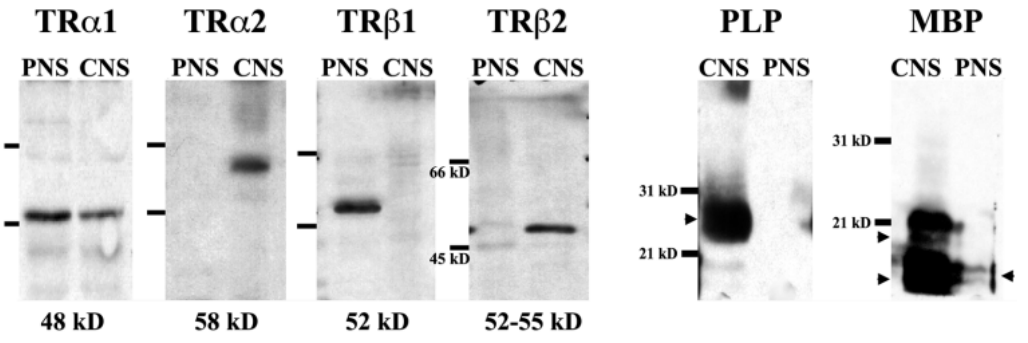

Fig. 7. Detection of thyroid hormone receptor proteins TRα1, TRα2, TRβ1 and TRβ2 and MBP and PLP proteins in myelin fractions of the central and peripheral nervous system. Myelin fractions were isolated and analyzed for immunoreactivity using western blotting and subtype specific antibodies as described in the Methods. While TRα1, TRα2 and TRβ2 antigens of appropriate size were detected in myelin fractions of the CNS, TRα1 and TRβ1 antigens were noted in myelin fractions of the PNS. PLP proteins were only detected in the myelin fraction of the CNS but not PNS while high levels of a 14-21 kDa MBP protein were found in myelin fractions of the CNS and low levels in myelin fractions of the PNS.

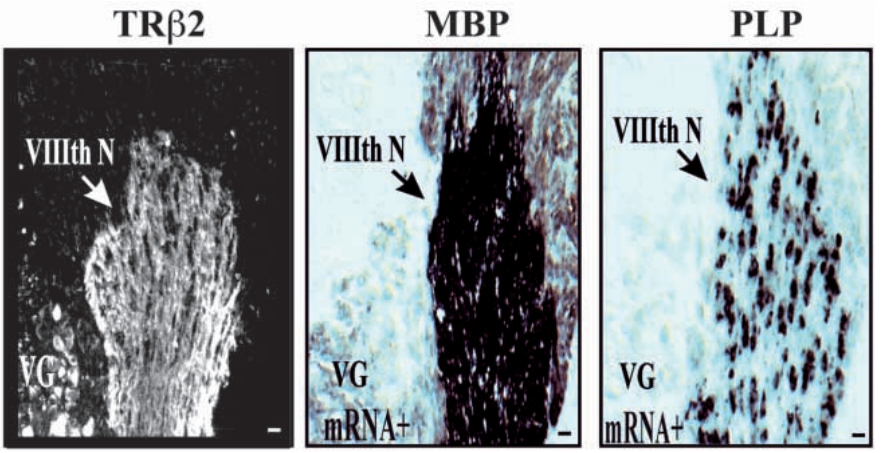

Fig. 8. TRβ2 expression in the central intradural part of the VIIIth nerve. TRβ2 proteins were detected in crossections of the cochlea at P10 using immunohistochemical analysis as described in the Methods. Neighboring sections were used for detection of MBP and PLP mRNA using in situ hybridization. Polyclonal antiserum to TRß2 showed strong immunoreactivity to the central part of the VIIIth nerve within the glial transition zone which at the same time showed strong hybridization signal to MBP and PLP. VIIIth N, eight nerve; VG, vestibular ganglion. Bar, 10 μm.

be important in improving our understanding of the distinct activities of thyroid hormone in the myelinogenesis process, as well as for all diseases related to thyroid hormone dysfunction (see for a review Waxman, 1985; Boyages and Halpern, 1993; Oppenheim et al., 1994; Bernal and Nunez, 1995). More especially, these data provide us with a first explanation for the frequent occurrence of severe peripheral neuropathy in human patients suffering from hypothyroidism (see for a review Perkins and Morgenlander, 1997).

The distinct expression of TRs in Schwann cells and oligodendrocytes of the cochlear nerve, also implies a dysfunction of individual TRs as a cause for hearing disabilities. In this context, the presumed special role of TRβs in the myelinogenesis of auditory fibers shown in the present study, may be of interest. In a number of earlier studies, TRα and TRβ, which act as transcription factors (Sap et al., 1986; Weinberger et al., 1986; Murray et al., 1988; Lazar and Chin, 1988; Hodin et al., 1989) were shown to be differentially expressed during embryonic development (Forrest et al., 1990; Yaoita et al., 1990, see for a review Bernal and Nunez, 1995). As with TR expression in the postnatal cochlea, shown in the present study, TRα gene expression was shown to occur in the rat brain during embryonic development, while the level of TRβ mRNA was found to be maximal by postnatal day 10, commencing at birth (Strait et al., 1992). The temporal association of distinct developmental steps with the rise of TRß receptors is supported by earlier observations (Yaoita and Brown, 1990), which demonstrated a marked increase in the expression of TRβ in tadpoles immediately preceding T3-induced metamorphosis (for a review see Oppenheim et al., 1994). In the inner ear, TRs have recently been described during embryonic and early postnatal development and the specific role of TRβ has been proposed to occur in the cochlea epithelia during the early postnatal period (Bradley et al., 1994). Surprisingly, TRβ-deficient mice mutants (ThrB–/–) were shown to exhibit deafness, despite no gross malformation of the organ of Corti (Forrest et al., 1996). The developmental alteration of the expression profile of TRβ mRNA in the cochlea, the distinct expression of TRβ in both the peripheral and central myelin, together with the localization of TRβs in the central intradural part of the VIIIth nerve, may suggest the participation or failure of TRß function in cochlear glial cells as a cause for deafness in ThrB–/– mutant mice. In this context, this discovery may also be of interest in terms of improving

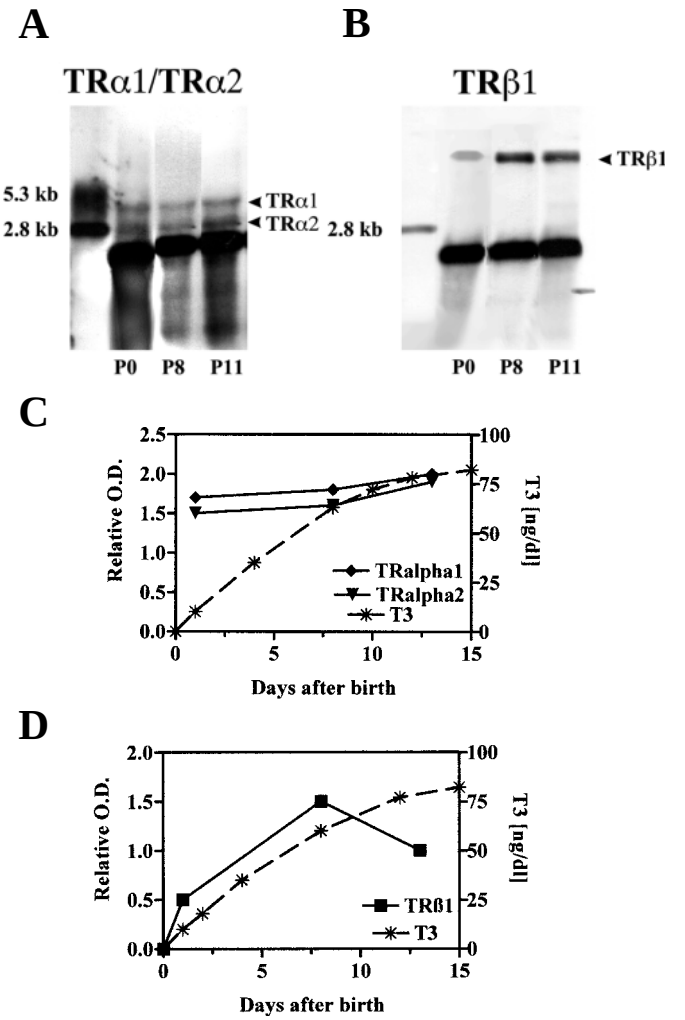

Fig. 9. Detection of TRα1, TRα2 and TRβ1 mRNA in cochleae of rats of the ages indicated. Tissue dissection and northern blotting was performed as described under Methods. (A) TRα1 and TRα2 were cohybridized revealing 2.6 kb mRNA for TRα2 and 5.0 kb mRNA for TRα1; (B) TRβ1 mRNA recognized mRNA of the expected size of 6 kb. In A and B actin riboprobes were cohybridized detecting 1.8 kb mRNA. (C,D) The slope of the expression profile of the distinct TR receptors is compared to the slope of triiodothyronine T3 plasma level (dotted line). Relative O.D. indicate average O.D.

our understanding of a distinct type of resistance to thyroid hormone (RTH-syndrome) in children homozygous for large gene deletions, including TRβ, which has been shown to induce deafness (Refetoff et al., 1993; Takeda et al., 1992).

In conclusion, the finding that TH can accelerate gene expression not only in oligodendrocytes but also in Schwann cells of the auditory tract, may lead to an improved understanding of the role of TH in the process of myelinogenesis. In the auditory system nerve conduction and impulse transmission from the cochlea to the brainstem can occur coincidentally with initial transduction of sound signals only in the presence of TH. In view of the possibility that auditory nerves, or even all nerves of placodal origin, may have a vulnerable developmental time span for a normal arborization of central fibers or patterning (as is the case in the visual system; for a review see Hockfield and Kalb, 1993; Cramer and Sur, 1995), retarded myelination of peripheral cochlea nerves, may be considered as participating in the deterioration or impairment of the auditory function.

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