Thyroid Hormone Deficiency Before the Onset of Hearing Causes Irreversible Damage to Peripheral and Central Auditory Systems

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Knipper, Marlies, Christoph Zinn, Hannes Maier, Mark Praetorius, Karin Rohbock, Iris Köpschall, and Ulrike Zimmermann. Thyroid hormone deficiency before the onset of hearing causes irreversible damage to peripheral and central auditory systems. J. Neurophysiol. 83: 3101–3112, 2000. Both a genetic or acquired neonatal thyroid hormone (TH) deficiency may result in a profound mental disability that is often accompanied by deafness. The existence of various TH-sensitive periods during inner ear development and general success of delayed, corrective TH treatment was investigated by treating pregnant and lactating rats with the goitrogen methimazole (MMI). We observed that for the establishment of normal hearing ability, maternal TH, before fetal thyroid gland function on estrus days 17–18, is obviously not required. Within a crucial time between the onset of fetal thyroid gland function and the onset of hearing at postnatal day 12 (P12), any postponement in the rise of TH-plasma levels, as can be brought about by treating lactating mothers with MMI, leads to permanent hearing defects of the adult offspring. The severity of hearing defects that were measured in 3- to 9-mo-old offspring could be increased with each additional day of TH deficiency during this critical period. Unexpectedly, the active cochlear process, assayed by distortion product otoacoustic emissions (DPOAE) measurements, and speed of auditory brain stem responses, which both until now were not thought to be controlled by TH, proved to be TH-dependent processes that were damaged by a delay of TH supply within this critical time. In contrast, no significant differences in the gross morphology and innervation of the organ of Corti or myelin gene expression in the auditory system, detected as myelin basic protein (MBP) and proteolipid protein (PLP) mRNA using Northern blot approach, were observed when TH supply was delayed for few days. These classical TH-dependent processes, however, were damaged when TH supply was delayed for several weeks. These surprising results may suggest the existence of different TH-dependent processes in the auditory system: those that respond to corrective TH supply (e.g., innervation and morphogenesis of the organ of Corti) and those that do not, but require T3 activity during a very tight time window (e.g., active cochlear process, central processes).

Introduction

One and one-half billion people worldwide potentially run the risk of suffering from iodine-deficiency disorders, including more than 600 million with goiter. As a clinical disorder, iodine deficiency is most damaging during the critical period of neural differentiation, causing severe, permanent brain abnormalities (Bernal and Nunez 1995; Oppenheimer et al. 1994). In addition to mental retardation, profound deafness is one of the noticeable features of congenital thyroid diseases, which may be associated with neurological cretinism or congenital goiter and is observed in Pendred syndrome (Trotter 1960) or in a resistance to thyroid hormone (RTH), respectively (Reftoff et al. 1967).

The thyroid hormone (TH) is necessary for normal development of the auditory system (Meyerhoff 1980; Ritter 1967). In rats TH is so far presumed to be active during the first 4 wk postnatal, when thyroxine deficiency leads to severe defects both of neuronal and morphogenetic cochlear processes (Uziel et al. 1983a, 1985a,b). Such defects include the abnormal persistence of afferent dendrites and absence of efferent innervation (Uziel et al. 1983b) or the absence of inner sulcus formation (Uziel et al. 1983a), caused by lack of cell regression in the great epithelial ridge (GER) of the immature Kölliker’s organ (Kölliker 1863). In addition to the postnatal TH-dependent period, the expression of thyroid hormone receptors (TR) in the inner ear of rodents before fetal thyroid gland function suggests a dependency on maternal TH during earlier periods of inner ear development (Bradley et al. 1994). Moreover, evidence indicates that TH is also active in the auditory system in adults (Goldey and Crofton 1998; Parving 1990). The mechanism by which the thyroid hormone exerts its manifold actions, whether for construction of a normal neuronal network in the developing brain (see for a review Bernal and Nunez 1995) or for inner ear development (Uziel 1985), has lost much of its scientific interest due to the common practice in developed countries of screening newborns for TH deficiencies, thus enabling TH replacement therapy to be carried out, if required. This has long been presumed to be a successful method for correcting mental retardation and hearing impairments (Kemp 1907). To explore the possibility of various TH-sensitive periods and their effect on inner ear development and to verify the success of normal inner ear development on delayed TH-supply, we studied the consequences of transient TH-deficient periods on the development of normal hearing in rodents. Pregnant and lactating rats were therefore treated with the goitrogen methimazole (MMI). MMI can cross the placenta and is observed in adult offspring of untreated (control) and MMI-treated dams. We observed that short periods of TH deficiency, when established within the critical developmental...
period before the onset of auditory function at postnatal day 12 (P12), result in permanent hearing impairment. TH-dependent processes were elucidated to occur during this time, which until now were not thought to be controlled by TH. On the other hand, well-known TH-dependent processes, such as the formation of the inner sulcus, synaptogenesis of efferent fibers of the organ of Corti, and myelin gene expression, remained unaffected on retraction of TH during this short critical time period, whereas they were damaged on prolonged periods of TH deficiency. These results may not only reveal novel targets for TH but also give new insights to the participation of TH in the development of the sensory system and brain.

**Methods**

**Drug administration and experimental design**

Wistar rats were purchased from Interfauna (Tuttlingen, Germany). The antithyroid drug methyl-mercapto-imidazol (MMI; 0.02%) was administered to the drinking water of pregnant females before embryonic day 17 (E17) to assure that TH levels were suppressed from the onset of fetal thyroid gland function onward, which in rats takes place on estra days 17–18 (Bernal and Nunez 1995; Calvo et al. 1990). The treatment was continued after birth and stopped between P3 and, maximum, P40. After discontinuation of MMI treatment, plasma levels of thyroxine (T4) and triiodothyronine (T3) returned to normal within 2–4 days and pups grew up under standard conditions. Hearing measurements of offspring of untreated (control) and MMI-treated dams were determined at 3-, 6-, or 9-mo of age. The neuronal and morphogenetic development of the inner ear was analyzed in tissue sections when animals were killed at the end of the experimental period. To study the effects of maternal TH, various couples of rats were paired for 3 or 8 days, separated and continuously treated with MMI either to E17 (during E3 to E17) or to specified days postnatally. The neuronal and morphogenetic inner ear development and the hearing function was analyzed in tissue sections of one to three offspring of three MMI-treated dams of each group (TH deficient for TH but also give new insights to the participation of TH in the development of the sensory system and brain. **T3/T4 determination**

Blood from the pups was collected when the animals were killed for isolation of the cochlea, whereas blood from pregnant dams was collected on retrobulbar puncture. The blood samples were allowed to clot and centrifuged at 1,000 rpm for 4 min. Serum was collected and frozen at −20°C for later use. Quantification of thyroxine (T4) or triiodothyronine (3,5,3’-triiodothyronine, T3) levels determined in rat serum was performed using a CIBA-Corning automated chemiluminescence system containing ACS-T4 and ACS-T3 ReadyPack Test reagents and a ACS 180 Chemiluminometer. The measurement is based on a competition of serum T3/T4 with T3, T4 analogues and T3, T4 antibodies.

**Tissue preparation**

Cochleae of postnatal or adult offspring of untreated (control) and MMI-treated dams were isolated and dissected as previously described (Knipper et al. 1997, 1998). Cochleae were fixed by immersion in 2% paraformaldehyde, 125 mM sucrose in 100 mM phosphate-buffered saline (PBS), pH 7.4, for 2 h, followed by overnight incubation in 25% sucrose, 1 mM protease inhibitor (Pefabloc, Boehringer, Mannheim, Germany) in PBS, pH 7.4. Cochleae of animals older than P10 were decalcified after fixation for 15 min to 2 h in Calex (Fisher Diagnostic, Fair Lawn, NJ). After overnight incubation, cochleae were embedded in O.C.T. compound (Miles Laboratories, Elkhart, IN). Tissues were then cryosectioned at 10 μm thickness, mounted on SuperFrost/plus microscope slides, dried for 1 h, and stored at −20°C before use.

**Immunocytochemical staining**

For immunohistochemistry, rat cochlea sections of offspring of untreated (control) or MMI-treated dams were thawed, permeabilized with 0.1% Triton-X-100 (Sigma) for 3 min at room temperature, preblocked with 1% bovine serum albumin in PBS, and incubated overnight at 4°C with antibodies. For double labeling studies, both antibodies were simultaneously incubated for identical time periods. We used antibodies to the 200 K neurofilament protein NF-200 as markers for afferent fibers (Berglund and Ryugo 1986; Knipper et al. 1997) and antibodies to synaptophysin as markers for presynapses in inner hair cell (IHC) and efferent fibers (Knipper et al. 1995, 1997; Wiechers et al. 1999). Polyclonal antisera for neurofilament 200 (N-4142) and monoclonal anti-synaptophysin (clone SVP-38) were purchased from Sigma. Primary antibodies were detected with either Cy3-conjugated secondary antibodies (0.35 μg/ml; Jackson Immuno Research Laboratories, PA) or fluorescein isothiocyanate (FITC)—conjugated secondary antibodies (1:100, Sigma). Sections were rinsed, mounted, and photographed using an Olympus AX70 microscope equipped with epifluorescence illumination. Photomicrographs were taken on Kodak TMY-400 film with 400 ASA.

**In vitro transcription of myelin basic protein (MBP) and proteolipid protein (PLP) cDNA**

MBP cDNA, cloned in pH322 by Roach et al. (1983) and PLP, cloned in pUC18 by Milner et al. (1985) were subcloned as described by Schraen-Wiemers and Gerfin-Moser (1993) and Lemke and Axel (1985). Subcloned cDNAs were kindly provided by M. Frank and M. E. Schwab, University Zurich, Switzerland. MBP cDNA encoded a 2.1 kb mRNA and PLP cDNAs encoded 3.2 and 1.6 kb mRNA. The digoxigenin-labeled RNA probes were transcribed from either T7 or T3 promoters for antisense, or sense transcripts in plBluescript in the presence of digoxigenin-UTP (Boehringer, Mannheim, Germany).

**Northern blot analysis of MBP and PLP mRNA**

Northern blot analysis was as recently described (Gestwicki et al. 1999; Knipper et al. 1998). The cartilaginous or bony capsule was carefully dissected from the cochlea of postnatal or grown pups of untreated (control) and MMI-treated dams. The opened spiral canals, including the modiolus around which they wind, were collected, immediately frozen in liquid nitrogen, and stored at −70°C. Brain stem, 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. Detection of mRNA was performed with a DIG System as recently described (Rueger et al. 1996). The effect of TH on mRNA levels was evaluated and semiquantified using isolated mRNAs from a similar number of cochleae as previously described (Knipper et al. 1998).

**Auditory brain stem responses**

Auditory evoked brain stem responses (ABR) to clicks were recorded in anesthetized rat pups or adult offspring (3–9 mo) of either untreated (control) or MMI-treated dams. Anesthesia was performed by intraperitoneal injection of 100 mg/kg ketamin hydrochloride (Ketamin 50 Curamed, CuraMED Pharma, Karlsruhe, Germany), 15 mg/kg xylazin hydrochloride (Rompun 290, Bayer Leverkusen, Germany), and 0.5 mg/kg atropin sulfate (Atropinsulfat, Braun, Germany). Generation of acoustic stimuli and subsequent recording of
evoked potentials were performed using a Phasics II System (Esaote-Biomedica, Italy). Acoustic stimuli, consisting of clicks were delivered monaurally at a rate of 1/1 s through a TDH-39 earphone (Telephonics) connected to a funnel fitted into the external auditory meatus. To record bioelectrical potentials, subdermal silver wire electrodes were inserted at the vertex (reference), ventrolateral to the measured ear (active) and contralateral to the measured ear (ground). After amplification and band-pass filtering (200 Hz to 2 kHz), electrical signals were averaged over 500 repetitions. The stimulus intensities used ranged from 120 dB peak equivalent SPL (peSPL) down to ABR threshold (defined as the lowest intensity at which a response could be recorded) in 5-dB steps. Approaching threshold, three replications were conducted. The thresholds were confirmed afterward by an independent observer and found not to differ by >5 dB.

Measurements of distortion product otoacoustic emissions (DPOAE)

For measurements of DPOAE, acoustic stimulation and measurement were performed by a PC-based system with an A/D converter board using Labview software, which also performed fast Fourier transformation (FFT) for data analysis. Pure tone continuous sound stimuli were generated and delivered on two separate channels using dynamic speakers (Beyer DT48). Sound levels were measured with a probe microphone (Bruel and Kjaer 4135) and a measuring amplifier (Bruel and Kjaer 2610). The speakers and microphone were coupled together, closed field into the animal’s ear canal, and the sound system calibrated with white noise (50 Hz to 20 kHz). DPOAEs were measured as cubic distortion products at the frequency 2f1-f2. For each frequency f1 (7, 12, or 18 kHz), the individual best ratio (BR) f2/f1 was determined using an intermediate sound level (60 dB SPL or higher, if necessary), varying the ratio from 1.06 to 1.44 in steps of 0.02. This ratio was used for the following input/output measurement. The sound level of f1 was kept at a constant 10 dB louder than the f2 level. Sound was initially presented at the lowest level and increased in steps of 5 dB. Recording windows were averaged so as to decrease the background noise to at least 10 dB below the level of emission, but not over more than 1,000 repetitions.

Experimental animals

The care and use of the animals reported in this study was approved by the University’s “special animal care” following the guidelines of the Declaration of Helsinki.

Results

Use of the MMI to control the plasma-TH level

Fetal and postnatal elevation of plasma-TH levels were examined by after treating dams with the goitrogen methimazole (MMI). To ensure that the plasma-TH level is suppressed from the onset of fetal thyroid gland function on day 17–18 (E17–E18) (Bernal and Nunez 1995; Calvo et al. 1990), pregnant females were treated with MMI before day 17 of embryonic development (E17). MMI treatment results in a severe depletion of tetraiodothyronine (T4) and triiodothyronine (T3) serum plasma levels in dams (data not shown) and induces a marked fetal T4 and T3 deficiency (Calvo et al. 1990). Due to placental transfer, MMI administration in dams leads to a complete depletion of serum plasma T4 and T3 levels in pups within 2–3 days (Knipper et al. 1999). Immediately after discontinuation of goitrogen-treatment on, e.g., P9, a rapid rise of the plasma-T3 level is observed in dams (not shown) and in pups (Fig. 1), with plasma-T3 levels returned to normal after a delay of 2–4 days, as shown in Fig. 1. Thus 69 ± 5 ng/dl T3 (Fig. 1, Hypo P9 + 4 day, mean ± SD, n = 6) was determined 4 days after discontinuing treatment with MMI on P9, which is not significantly different from 73 ± 11 ng/dl T3 in untreated control animals of the same age at P13 postnatal (Fig. 1, control 13 days, n = 4; P > 0.1). A T3 level slightly above normal was occasionally observed before the T3 level plateau (Fig. 1, Hypo P9 + 3 day). Three months after discontinuing MMI, the T3 level reached 67.3 ± 5.6 ng/dl T3 (Fig. 1, Hypo P9 + 3 mo, n = 9), which is not significantly different from T3 levels in untreated 3-mo-old animals with 70.6 ± 7.7 ng/dl T3 (Fig. 1, control 3 mo, n = 5; P > 0.1).

Use of the MMI to control the plasma-TH level

FIG. 1. Thyroid hormone (TH) levels after discontinuation of methyl-mercapto-imidazol (MMI). Dams were treated with MMI until pups were postnatal day P9 (Hypo P9). After discontinuation of MMI (Hypo P9 + 1 day to Hypo P9 + 4 day) the triiodothyronine (T3) levels returned to normal within 2–4 days. After 4 days T3 levels were similar to same aged controls, shown for pups at P13 (Control P13). Three months after discontinuation of MMI (Hypo P9 + 3 mo) the T3 levels were not significantly different from similar aged control animals (Control 3 mo). Dark bars, offspring of MMI-treated animals; light bars, offspring of untreated control animals; ** mean ± SD, P > 0.05; n = 4–8.

Short periods of TH deficiency before the onset of hearing ability lead to elevated hearing thresholds

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Results

Use of the MMI to control the plasma-TH level

Fetal and postnatal elevation of plasma-TH levels were examined by after treating dams with the goitrogen methimazole (MMI). To ensure that the plasma-TH level is suppressed from the onset of fetal thyroid gland function on day 17–18 (E17–E18) (Bernal and Nunez 1995; Calvo et al. 1990), pregnant females were treated with MMI before day 17 of embryonic development (E17). MMI treatment results in a severe depletion of tetraiodothyronine (T4) and triiodothyronine (T3) serum plasma levels in dams (data not shown) and induces a marked fetal T4 and T3 deficiency (Calvo et al. 1990). Due to placental transfer, MMI administration in dams leads to a complete depletion of serum plasma T4 and T3 levels in pups within 2–3 days (Knipper et al. 1999). Immediately after discontinuation of goitrogen-treatment on, e.g., P9, a rapid rise of the plasma-T3 level is observed in dams (not shown) and in pups (Fig. 1), with plasma-T3 levels returned to normal after a delay of
Elevated hearing thresholds are not caused by depletion of maternal TH before E17

Because thyroid hormone receptors are expressed in the fetal otocyst before the onset of fetal thyroid gland function at E17 (Bradley et al. 1994), we thought to confirm that the observed elevation of hearing thresholds was not a result of the depletion of maternal plasma-TH levels. To analyze the effect of maternal TH, the plasma-TH level of dams was suppressed with MMI either 3 days after mating until E17 (E3 to E17) or 3, 8, or 17 days after mating until P12 (E3 to P12, E8 to P12 or E17 to P12). Regardless of whether dams were treated with MMI from E3, E8, or E17 onward, a morphologically intact cochlea and a normal organ of Corti at the developmental level of the Kölliker’s organ (Kölliker 1863) was observed in pups at day 3 postnatal (n = 6, no deviation, not shown). Offspring of mothers treated with MMI exclusively between E3 and E17 generated hearing thresholds similar to those of controls (Table 2). Furthermore, regardless of whether animals were treated from E3, E8, or E17 onward to P12, the elevated hearing thresholds in 3-mo-old animals were not significantly different from each other (Table 2). It may be concluded from these observations that maternal TH has no direct influence on the development of the auditory system.

Normal morphogenesis and innervation of the organ of Corti in animals with elevated hearing thresholds

As the first step in analyzing the molecular basis for elevated hearing thresholds in animals that had undergone a TH-deficient period before the onset of hearing (E17 to P9), the gross morphology and innervation of the cochlea of 3- to 9-mo-old offspring of untreated (control) and MMI-treated dams was studied. The inner sulcus formation and the establishment of mature afferent and efferent contacts below outer hair cells (OHCs) are known to be controlled by TH (Uziel 1985). The mature innervation pattern can be visualized by neuronal marker proteins. For double-labeling studies we used antibodies directed against the 200 K neurofilament protein NF-200, which stain afferent fibers and antibodies against synaptophysin, which stain presynapses as IHCs and efferent fibers. The apical to basal cochlea turn of the left and right ear of at least three animals from each individually treated group were analyzed twice. The results are demonstrated for 6-mo-old offspring of untreated mothers (control) in comparison to 6-mo-old offspring of dams that were treated with MMI until the pups reached P9 (Fig. 3). Toluidin blue-stained sections of the...
cochlea did not reveal any significant structural differences between control and treated animals along the cochlear tonotopic axis; both had a normally developed inner sulcus (Fig. 3A, n = 8). We occasionally observed a dislocation of the tectorial membrane (Fig. 3A, Hypo P9, Tec), in both control and treated animals. Thus no information about the structure of the tectorial membrane can be obtained from cryosectioned cochlea tissue. In addition, strong synaptophysin-immunopositive efferent fibers projecting to OHCs and IHCs and synaptophysin-immunopositive efferents opposite to IHC (open arrowhead) and OHCs (closed arrowhead) (B). Typically less-dense NF-200-immunopositive afferents opposite to OHCs (closed arrowhead), whereas intensive stained NF-200-immunopositive afferent fibers project to IHCs (open arrowhead; C). OHC, outer hair cells; IHC, inner hair cells; Tec, tectorial membrane (bar = 10 μm).

Elevated hearing thresholds are accompanied by impaired active cochlear mechanics and retarded maturation of central auditory processes

Because normal morphogenesis and innervation of the organ of Corti was observed in hearing-impaired offspring of MMI-treated dams, we sought for other targets of TH action in the cochlea. First, DPOAEs, which reflect active cochlear mechanisms, were investigated. DPOAEs are dependent on the presence of a normal endocochlear potential and on both intact mecanoelectrical and electromechanical transduction on the level of OHCs. In adult control animals, DPOAEs at low and moderate sound levels (<80 dB SPL) were detected at all frequencies measured (7, 12, and 18 kHz, Fig. 4A, circles, n = 3) and disappeared immediately (5 min) after death (Fig. 4A, triangles). In age-matched offspring, the mothers of which had been treated with MMI until the pups were P10 or older (n = 8), no DPOAEs were detected above the level of setup distortion as shown for animals treated with MMI until P13 (Fig. 4B, triangles, n = 3). Most interestingly, normal DPOAE levels were found in animals that experienced a shorter TH-deficient period (E17 until P3 or E17 to P5; Fig. 4B, circles, n = 3, Table 3B). Moreover, offspring of dams treated with MMI until P8 could be divided into two groups, those with normal and those with impaired DPOAEs (Table 3B), indicating the extraordinarily short time period during which TH can successfully influence the establishment of intact active cochlear functions.

Although an effect on DPOAEs may reflect changes in developmental processes within the cochlea, the detection of auditory brain stem interpeak intervals provides information on the degree of maturation of central auditory processes, including myelination and maturation of synapses and dendrites. The interpeak intervals are determined as latencies from four major waves, generated as a consequence of the sequential activation of the VIIIth nerve (wave I), the cochlear nuclei (wave II), the superior olive (wave III), and the inferior colliculus (wave IV) (Jewett and Romano 1972). I–IV interpeak intervals from ABR were measured in 3- to 9-mo-old offspring of untreated dams (control) and were compared with ABR-interauditory intervals of offspring from the same age that endured short periods of TH deficiency from the onset of thyroid gland function until P10 (not shown), P8, P10, P12, and P14 (Fig. 5). The latency time of ABR waves (Fig. 5, Latency ms) is depicted against the individual equivalent sound pressure level used for generation of the response (Fig. 5, dB peSPL). Mature ABRs in adult animals are characterized by latencies that increase only slightly with declining sound pressure levels.
(Fig. 5, control). A TH-deficient period between the onset of thyroid gland function at E17 up to P5 (not shown) or P8 (Fig. 5) was seen to result in elevated hearing thresholds, but normal ABR latencies. This is demonstrated by a parallel decline of latencies in hearing-impaired (P8) and control animals (Fig. 5, compare slope of the line of P8 and control for wave I to IV). However, by extending the TH-deficient period during the critical time period until P10, P12, P13, and P14, increasing ABR latencies are obtained with each further day of TH postponement. This can be detected in a gradual shift toward higher ABR latencies (Fig. 5, compare slope of the line of P10 to P14 for wave I to IV). These results are summarized in Table 3, which documents the ABR latencies for waves I to IV (ms) generated at 60, 70, and 80 dB pSPL. For comparison, the table includes the distinct hearing thresholds (Table 3A) and DPOAEs (Table 3B) of the differently treated groups. In animals that underwent a TH-deficient period until P3 (not shown) or P5 (Table 3A, P5), the observed elevated hearing thresholds were correlated with normal DPOAE levels (Table 3B, P5) and normal ABR latencies (Table 3C, P5). Animals with a delayed rise of plasma-TH level until P8 generated normal ABR latencies (Table 3, P8, boldface), although some of these animals already displayed impaired DPOAEs. The delay in the rise of plasma-TH levels beyond P10 led to elevated thresholds (Table 3A), absence of DPOAEs (Table 3B), and significantly enhanced ABR latencies when compared with controls (Table 3C, P10, P12, P13, and P14). The delay with which TH obviously gradually affects more central auditory nuclei became evident from the latencies in the higher nuclei, generating wave II and IV, which during a prolonged delay in the rise of plasma-TH levels were less affected (Table 3C).

### Table 3. Comparison of (A) hearing thresholds, (B) presence/absence of active cochlear mechanics (DPOAEs) and (C) ABR latencies (Wave I–IV) in adult offspring of untreated dams (Control) or adult offspring of dams treated with MMI from <E17 to indicated postnatal days (P5 to P14)

<table>
<thead>
<tr>
<th>dBp SPL</th>
<th>Control</th>
<th>P5</th>
<th>P8</th>
<th>P10</th>
<th>P12</th>
<th>P13</th>
<th>P14</th>
</tr>
</thead>
<tbody>
<tr>
<td>A Threshold, dBpSPL</td>
<td>25 ± 9 (16)</td>
<td>46 ± 5b</td>
<td>56 ± 13b</td>
<td>63 ± 9b</td>
<td>76 ± 10b</td>
<td>72 ± 7.0b</td>
<td>81.0 ± 10b</td>
</tr>
<tr>
<td>B DPOAE</td>
<td>+ + + (3)</td>
<td>+ + + (3)</td>
<td>+ + (2)</td>
<td>No signal (2)</td>
<td>No signal (2)</td>
<td>No signal (2)</td>
<td>No signal (2)</td>
</tr>
<tr>
<td>C Wave I</td>
<td>6.0</td>
<td>1.0 ± 0.12</td>
<td>1.27 ± 0.12</td>
<td>1.3 ± 0.18</td>
<td>1.3 ± 0.06</td>
<td>1.54 ± 0.28</td>
<td>1.72 ± 0.19</td>
</tr>
<tr>
<td>Wave II</td>
<td>6.0</td>
<td>2.04 ± 0.08 (9)</td>
<td>2.03 ± 0.07</td>
<td>2.13 ± 0.11</td>
<td>2.15 ± 0.09</td>
<td>2.55 ± 0.11</td>
<td>2.51 ± 0.24</td>
</tr>
<tr>
<td>Wave III</td>
<td>6.0</td>
<td>1.93 ± 0.14 (7)</td>
<td>2.01 ± 0.18</td>
<td>2.09 ± 0.19</td>
<td>2.19 ± 0.28</td>
<td>2.24 ± 0.18</td>
<td>2.37 ± 0.18</td>
</tr>
<tr>
<td>Wave IV</td>
<td>6.0</td>
<td>2.07 ± 0.09 (9)</td>
<td>2.76 ± 0.19</td>
<td>2.75 ± 0.18</td>
<td>3.11 ± 0.26</td>
<td>3.13 ± 0.31</td>
<td>3.33 ± 0.38</td>
</tr>
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</table>

Values are means ± SD; number in parentheses is number of animals. DPOAE, distortion product otoacoustic emission; ABR, auditory evoked brain stem response. *ABR thresholds, DPOAEs and ABR latencies are measured as described under methods. Note the increase of hearing defects during each additional day of TH deficiency within the critical time prior to hearing (bold). **n = 6–16, P < 0.05, *n = 4–9, P > 0.1, †n = 3–13, P < 0.05. Wave I (ABR latency, ms) generated due to activation of the VIIIth nerve. Wave II (ABR latency, ms) generated due to activation of the cochlear nuclei. Wave III (ABR latency, ms) generated due to activation of the superior olive. Wave IV (ABR latency, ms) generated due to activation of the inferior colliculus.
Elevated ABR latencies are not caused by developmental retardation

Based on interpeak intervals of ABR, the maturation of central auditory processes is completed around the end of the third month in rodents (Moore 1985). To confirm that the observed elevated latencies in animals treated with MMI are not a result of retarded maturation of central auditory processes, which may improve during later development, we analyzed ABR latencies in offspring of MMI-treated dams between 3 and 9 mo of age. Auditory brain stem responses were induced by 80 dB peSPL click signals. As shown in Table 4 for animals that underwent a TH-deficient period until P10, there was no further improvement of ABR latencies between 3 and 9 mo of age. This confirms that elevated ABR-interpeak intervals that arise as a result of a short TH-deficient period before the onset of hearing are probably permanent.

Table 4. Elevated ABR latencies, induced by a TH-deficient period between E17 and P10, do not recover over time*

<table>
<thead>
<tr>
<th>Wave of ABR Potential</th>
<th>Latency, ms</th>
<th>Student’s t-test</th>
<th>n</th>
</tr>
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<tbody>
<tr>
<td>Wave I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 mo</td>
<td>1.27 ± 0.14</td>
<td>P &gt; 0.62</td>
<td>7</td>
</tr>
<tr>
<td>9 mo</td>
<td>1.24 ± 0.13</td>
<td></td>
<td>7</td>
</tr>
<tr>
<td>Wave II</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 mo</td>
<td>2.11 ± 0.21</td>
<td>P &gt; 0.64</td>
<td>7</td>
</tr>
<tr>
<td>9 mo</td>
<td>2.07 ± 0.09</td>
<td></td>
<td>8</td>
</tr>
<tr>
<td>Wave III</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 mo</td>
<td>2.91 ± 0.21</td>
<td>P &gt; 0.66</td>
<td>7</td>
</tr>
<tr>
<td>9 mo</td>
<td>2.96 ± 0.24</td>
<td></td>
<td>8</td>
</tr>
<tr>
<td>Wave IV</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 mo</td>
<td>3.98 ± 0.35</td>
<td>P &gt; 0.46</td>
<td>7</td>
</tr>
<tr>
<td>9 mo</td>
<td>3.84 ± 0.38</td>
<td></td>
<td>8</td>
</tr>
</tbody>
</table>

Values in Latency are means ± SD; n is number of animals. For abbreviations, see Tables 1 and 3. * ABR latencies of Wave I to Wave IV (ms) were determined in 3- and 9-mo-old offspring of MMI-treated dams. Dams were treated with MMI until P10 of the pups.

Elevated hearing thresholds are not due to retarded myelin gene expression

A retardation of the speed of auditory brain stem responses may be caused by hypomyelination of auditory nerves, resulting from incomplete myelin gene expression, which itself may be associated with TH deficiency (see for a review Oppenheimer et al. 1994). Myelin gene expression in cochlea, brain stem, inferior colliculus, and the auditory cortex of 3-mo-old offspring of untreated and MMI-treated dams was therefore analyzed. In Northern blot experiments MBP mRNA (Fig. 6, MBP) and PLP mRNA (Fig. 6, PLP) was detected in the distinct auditory tissues. Semiquantification of the mRNA levels was carried out with laser densitometry. As shown in Fig. 6 for untreated animals and animals that had undergone a TH-deficient period (E17 to P13), no significant difference in the mRNA levels of either MBP or PLP in any of the tested tissues could be detected. Moreover, we could find no differences in MBP and PLP mRNA level in the distinct auditory nuclei, regardless of whether they experienced a TH-deficient period postnatally until P8, P10, P12, or P14 (not shown). Not even the total absence of TH up to P28 led to any detectable change in myelin gene mRNA level in the peripheral or central auditory system of 3-mo-old animals (not shown). Figure 6 illustrates the results run in a single gel, and similar results were obtained in two independent experiments.

Novel TH-dependent processes in the auditory system that do not allow any postponement of TH supply

Temporal sequence of failure of distinct cochlear and auditory brain stem processes due to prolongation of the TH-free period before hearing function is shown for individual animals in Fig. 7. Comparison can be made between the threshold shifts (Fig. 7A), plasma-T3 levels (ng/dl; Fig. 7B) and the individual hearing impairment (Fig. 7C). As
previously reported (Knipper et al. 1999), the presence of triiodothyronine (T3) only from the onset of fetal thyroid gland activity (E17) until P12 (seen in curve 3 in Fig. 7B), leads to a normal hearing threshold that is not different from hearing thresholds in untreated control animal (Fig. 7A, compare bar 3 and 4). Animals with a restricted rise of plasma-T3 levels between P1 and P7 (Fig. 7B, curve 2) or those with depleted plasma-T3 levels (Fig. 7B, curve 1) failed to generate normal hearing function (Fig. 7A, compare bar 1 and 2). The delay in the rise of plasma-TH levels from the onset of fetal thyroid gland activity onward until P3, P5, P8, P9, P10, P12, or P14 (Fig. 7B, curve 5 to 10) leads to gradually, permanently elevated hearing thresholds (Fig. 7A, bar 5 to 10). The severity of hearing defects increases with each additional day of MMI-induced T3 absence during this crucial period (Fig. 7C). This is reflected by elevated thresholds (Fig. 7C, bar 5), which were subsequently accompanied by a failure of active cochlear functions (Fig. 7C, bar 7) and a decline in the speed of brain stem responses (Fig. 7C, bar 8–10). Only when T3 is absent...
for more than 3 wk postnatally, cochlear morphogenesis and innervation is permanently impaired (Fig. 7C, immature Org. Corti).

**Discussion**

The discovery of a thyroid extract therapy to cure mental diseases that are associated with cretinism by Murray and its description by Sir William Osler dates back to 1891 (Murray 1891; Osler 1889). The first indications that TH is also essential for auditory functions were soon to follow, and replacement therapy with thyroid extracts was reported to have the capability of improving hearing defects as well as the growth and mental development of infants suffering from cretinism (Kemp 1907). Approximately 60 yr later, this concept was queried and the proposal made that under distinct conditions, hearing dysfunction may be permanent despite early treatment with thyroid hormone (Deol 1973). However, later findings defined the corrective effects of thyroxine for various developmental processes in further detail (Uziel et al. 1985a,b). Progressive irreversible morphogenetic or neuronal damage to the cochlea was found in hypothyroid rats only after the first two postnatal months (Uziel et al. 1985a). Because the developmental stage of 1-yr-old infants corresponds approximately to that of 1- to 2-mo-old rats (Eggermont et al. 1991; Teas et al. 1982), TH-replacement therapy in humans, if begun before 1 yr of age in infants, has been assumed to successfully compensate hearing loss as a result of TH deficiency.

**Specificity of MMI effects**

Using methimazole (MMI) instead of propylthiouracil (PTU) (Uziel et al. 1983a,b) as goitrogen, we recently confirmed the irreversible neuronal and morphogenetic damage of the cochlea when plasma-TH levels are suppressed from \(<E17\) until \(>P28\) (Knipper et al. 1999). Here we document the irreversible elevation of hearing thresholds when shorter TH-deficient periods (using MMI) are induced between the onset of fetal thyroid gland activity at \(E17\)-\(E18\) and the onset of hearing at \(P12\). MMI and PTU are common goitrogens that inhibit thyroid peroxidase-catalyzed thyroid hormone formation. Both drugs have been used safely for decades to suppress plasma-TH levels in animals and humans (Becks and Burrow 1991; Lind 1997). Neither the administration of MMI before \(E17\) (present study) nor the administration of MMI after \(P1\) or \(P2\) (Knipper et al. 1999) had any effect on the development of normal hearing. Thus MMI induces hearing loss only when the MMI-induced T3-deficient period spans the critical developmental period before the onset of hearing. Within this critical period we can also observe a deterioration of hearing, which appears to reflect the subsequent failure of TH-dependent steps along the peripheral-centripetal axis of the auditory system. We presume from these observations that the effects on inner ear development by MMI are mediated by a specific effect on T3 synthesis, rather than due to a cochlear sensitivity to a presumptive MMI toxicity especially during this limited critical period.

**Role of maternal TH for hearing function**

Plasma-TH level in dams declines within 2–3 days after MMI treatment, similar to that described for the pups (Knipper et al. 1999). Because mating was limited to 3 days, the administration of MMI to dams from the third day after mating is presumed to lead to depletion of the plasma-TH level in dams sometimes between \(E3\) and \(E6\). The absence of TH in dams between \(E3\) and \(E17\) (Table 2) would therefore lead to a TH-free period at least between \(E6\) and \(E17\) (Table 2). In another group, administration of MMI to pregnant dams from \(E3\) to \(P12\), \(E8\) to \(P12\), and \(E17\) to \(P12\) (Table 2) guarantees that TH is suppressed during the next developmental period, when the pups are able to produce TH via their own thyroid gland activity from \(E17\) onward. The analysis of both groups thus covers the developmental period when \(\alpha\)- and \(\beta\)-thyroid hormone receptor expression was observed in the neonatal otocyst from about \(E14\) onward (Bradley et al. 1994), the time maternal TH is presumed to act. The decline of maternal TH between \(E3\) and \(E17\) is of no consequence for the development of hearing, because adult rats have similar thresholds than control rats (Table 2) and because hearing thresholds in offspring of MMI-treated dams from \(E3\) to \(P12\), \(E8\) to \(P12\) and \(E17\) to \(P12\) are similar (Table 2). This implies that the neonatally expressed TR receptors in the otocyst (Bradley et al. 1994) act in hormone-independent pathways, as described for other tissues (Helmer et al. 1996). This result is in good agreement with recent findings describing the lack of effects of thyroid hormone on late fetal rat brain development (Schwartz et al. 1997). Neither gene expression nor cerebellar morphogenesis were altered in the absence of fetal TH (Schwartz et al. 1997).

**Novel TH-dependent processes during the critical developmental time period before hearing function**

Our data imply that the observed hearing impairment is obviously not due to a failure of the classical TH-dependent processes, the formation of the inner sulcus and maturation of efferent and afferent innervation (Fig. 3), both of which arise after prolonged TH-deficient periods (Deol 1973; Uziel 1985). This suggests the existence of additional TH-dependent processes during auditory system development and indicates that the auditory system reacts different to a corrective TH supply. Although morphogenesis and innervation of the cochlea apparently do respond to corrective TH supply, other TH-dependent processes require T3 activity during a very tight time period. The increase in hearing loss after prolongation of a TH-deficient period is reflected first in affected thresholds (Figs. 2 and 7A), then in affected thresholds and impaired active cochlear function (Figs. 2 and 4), and finally in affected thresholds, impaired active cochlear function and elevated brain stem latencies (Figs. 2 and 5, Table 3). This implies that TH deficiency during this critical period of development affects individual sections along the centripetal axis of the auditory system step by step. This concept is supported by the observed tendency of higher order brain stem nuclei to be less affected when T3-deficient periods are prolonged (Table 3C). These differences are probably connected to the time at which TH usually acts in these areas during development. Therefore the various grades of hearing impairment appear to reflect different targets of TH in the auditory system, starting from the periphery in a centripetal direction. Elevated hearing thresholds in animals that have undergone TH deficiency from the onset of thyroid function (\(E17\)) onward up to \(P3\) and \(P5\) (Fig. 2, Table 3A) may reflect the earliest TH activities within the
organ of Corti. In this context, it will be interesting to define the effects of TH on potassium channels in hair cells, whose expression was recently shown to be accelerated by TH (Ruesch et al. 1999). However, the possibility of TH-dependent changes affecting cochlea ultrastructure of the cytoskeleton (Poddar et al. 1996) or the expression or posttranslational modification of functional important proteins (Tosic et al. 1992) should also be considered.

In all animals that underwent a delay in the rise of TH until P8 or thereafter, we also observed a loss of DPOAEs (Fig. 4B, Table 3B). DPOAEs are sound emissions from the ear, which are produced by pairs of tones that interact nonlinearly to produce extra tones (Patuzzi 1985). The active mechanisms in the cochlea are driven by hair cell electromotility, which is in turn dependent on a functional mechanoelectrical transduction of acoustic stimuli, as well as on intact endocochlear and membrane potentials (for a review, see Dallos and Evans 1995). The observed impairment of DPOAEs may thus be due either to impaired mechanoelectrical or electromechanical transduction or impaired endocochlear potential (EP), one of the driving forces for electromotility (for a review see, Patuzzi 1996). Although thyroid hormone receptors have been found to be expressed in OHCs (Knipper et al. 1998; Lautermann and Ten Cate 1997), so far neither of the processes that are the driving forces for electromotility have been shown to be controlled by TH. Future studies should therefore be aimed at discovering TH-dependent steps that are responsible for the loss of DPOAEs in animals that sustained TH-deficient periods before hearing.

A decrease in speed of auditory brain stem responses may be caused by hypomyelination of the auditory nerve. There are several reports that underline the possibility that parts of the myelination process, including myelin gene expression, may be under the control of TH (for a review see Oppenheimer et al. 1994). We recently observed that the expression of myelin genes in the peripheral and central part of the intradural segment of the VIIIth nerve began simultaneously and before cochlea function; expression rapidly achieved its maximal levels around P12, the day on which cochlea function commences (Knipper et al. 1998). It was shown that TH is responsible for the immediate saturation of myelin gene expression (Knipper et al. 1998). The part played by TH-dependent processes in improvement hearing sensitivity may thus include the timely expression of myelin gene expression before the onset of hearing at P12 (Knipper et al. 1998) and thereby may be a prerequisite for the nerve conduction and nerve impulse transmission along the auditory tract (Knipper et al. 1998), the driving forces for the subsequent maturation steps of central auditory projections (Eggermont et al. 1991; Moore et al. 1995). Maturation processes in the central auditory system include the increase in myelin sheaths and the maturation of central synapses and dendrites (Eggermont et al. 1991; Moore et al. 1995; Shah et al. 1978). Indeed, similar to the visual system, various studies support the notion that the timely onset of auditory experience is essential for the development of intact maturation of central auditory processes (Ptok and Ptok 1996; Walger et al. 1993). In the present study elevated latencies were observed in animals that had sustained TH-deficient periods until P10, but not in those with shorter TH-deficient periods (Table 3). Elevated latencies were observed, although no reduction of myelin gene expression along the auditory tract in these animals was detected (Fig. 5). Considering that the delayed activity of TH in these animals definitely leads to retarded myelin gene expression, as shown previously (Knipper et al. 1998) and under the assumption that the saturation of the myelin gene expression may indeed be a prerequisite for auditory experience to promote central maturation steps, the observed enhanced ABR latencies could be an indirect consequence of impaired cochlear function and retarded onset of auditory experience during development. In the light of these results, the possible connection between the observed impairment of the cytoarchitecture of the neocortex and cerebellum and thyroid hormone deficiency should be reconsidered (Berbel et al. 1993; Eayrs 1971; Graves and Hawkes 1990; Legrand 1967).

Comparison of the critical period of the inner ear in rats and humans

After MMI treatment was stopped, T3 levels returned to normal within 2–4 days (Fig. 1). After the delay in the rise of plasma-T3 levels from the onset of fetal thyroid gland function (E17) up to P3, P5, and P8, T3-levels are therefore back to normal before P12, which marks the end of the TH-dependent period (Knipper et al. 1999) and the onset of hearing function (Rubel 1978; Shah et al. 1978). Based on the degree of cochlear maturation in rats (Lavigne-Rebillard and Pujol 1987) and humans (Johansson et al. 1964; Moore et al. 1995), as well as the precise time at which brain stem responses and active cochlear functions can be registered in rats (Geal-Dor et al. 1993; He et al. 1994; Jewett and Romano 1972) and humans (Birnholz and Benacerral 1983; Bonfils et al. 1988; Ponton et al. 1996; Starr et al. 1977), P12 in rats corresponds to the developmental stage of the auditory system at approximately the 29th embryonal week in humans. Thus the observed hearing defects in transiently TH-deprived animals, which have been shown in the present study to return to normal plasma-T3 levels before P12, confirm that the developmental stage at which a TH-replacement therapy in humans should be most successful in avoiding hearing impairment will be long before birth, even before the 29th embryonal week (see Fig. 7, B and C). The benefits of TH-replacement therapy for newborns to improve hearing ability and its exact timing should therefore be urgently reconsidered. Current recommendations for sufficient TH and iodine levels during pregnancy may have to be altered because even short, transient periods of TH and iodine deficiency during the critical period between the onset of fetal thyroid gland function (approximately the 10th embryonal week) and the onset of the first auditory brain stem responses within the 29th embryonal week (Moore et al. 1995), may be suggested to be the cause of various degrees of congenital hearing deficiencies.

Some genetic diseases related to thyroid dysfunction are accompanied by hearing loss but, similar to findings demonstrated here, showed normal cochlear morphogenesis and innervation (Biebermann et al. 1997; Green et al. 1988; O’Malley et al. 1995; Utiger 1995). The various degrees of hearing impairment observed in multiple forms of genetic and acquired diseases related to thyroid dysfunction (Bernal and Nunez 1995; Biebermann et al. 1997; Meyerhoff 1980; Op-
penheimer et al. 1994; Refetoff et al. 1967; Trotter 1960) should therefore be reconsidered in the context of the results presented here.

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