Prevention of Auditory Dysfunction in Hypothyroid *Tshr* Mutant Mice by Thyroxin Treatment During Development

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Received: 11 September 2000; Accepted: 11 April 2001; Online publication: 1 August 2001

ABSTRACT

Based on previous work, it is clear that genetically hypothyroid *Tshr*^{hyt} mutant mice are congenitally deaf [O'Malley et al. (1995) *Hear. Res.* 88: 181–189, Sprinkle et al. 2001b, *J. Assoc. Res. Otolaryngol.* DOI: 10.1007/s101620010077]. However, the extent to which auditory development is dependent on the availability of thyroxin (T₄) during specific developmental stages is unknown. The aim of this study was to determine the relative importance of prenatal and postnatal thyroxin on the ontogeny of hearing in the *hyt* mouse. Experimental hypothyroid subjects were offspring of *hyt/hyt* breeders implanted with T₄ or placebo controlled-release pellets 14 days prior to mating. Pups received T₄ or saline placebo injections from birth through postnatal day 14 (P14) or the time of testing on P28. In the absence of exogenous T₄ replacement, very high stimulus levels (>80 dB SPL) were required to elicit responses. Remarkably, T₄ treatment confined to the postnatal period failed to significantly improve auditory function relative to untreated animals, while response thresholds, latencies, and amplitudes of mice born to dams that received T₄ during pregnancy were significantly improved relative to both of the untreated groups. Response thresholds were improved somewhat when maternal T₄ replacement was followed by treatment during the first 14 days of life, and animals treated throughout prenatal and postnatal life were

comparable to those of age-matched euthyroid individuals. Findings from this study show that treatment of *hyt/hyt* mice with exogenous T₄ significantly attenuates hypothyroid-induced otopathology in a developmental-stage-dependent manner. In addition, we demonstrate that postnatal development is critically dependent on prenatal exposure to thyroxin and that the critical window of T₄ dependence extends throughout development.

Keywords: hypothyroidism, development, hearing, deafness, maturation, thyrotropin

INTRODUCTION

It is generally understood that the severity of hypothyroidism-induced impairment of auditory system development in mammals is proportional to the onset and duration of thyroid hormone deficiency. When initiated in a mature animal, thyroid hormone deficiency generally produces a mild form of otopathology that is typically responsive to thyroxin (T₄) replacement therapy (Deol 1973; Hébert et al. 1985a, b). Hypothyroidism arising early in development, on the other hand, characteristically produces more severe auditory deficits that are typically refractory to replacement therapy (Deol 1973; Meyerhoff 1979; Uziel et al. 1983, 1985; Hébert et al. 1985a, b). The main body of existing data suggests that the auditory system is most sensitive to thyroid hormones from the beginning of autonomous secretion through the age that the first responses to airborne sounds can be elicited (Deol 1973; Hébert et al. 1985b). In rodents, as in most

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altricial mammals, autonomous secretion begins late in the prenatal period (Jacobson and Brent 1959; van Heyningen 1961) and the first responses to airborne sounds occur between postnatal days 9 and 14 (Alford and Ruben 1963; Mikaelian and Ruben 1965; Ehret 1976), coincident with the final stages of cochlear cyto-differentiation (Kikuchi and Hilding 1965; Mikaelian and Ruben 1965; Shnerson et al. 1982). Consistent with that view, when hypothyroidism is induced in rats and mice after P10 or so—just around the time that evoked potentials are first observed in altricial rodents—ensuing peripheral auditory pathology is relatively minor. More severe anatomical abnormalities occur when hypothyroidism is initiated earlier in postnatal life or, especially, prior to the time that autonomous secretion of thyroxin occurs in developing fetuses, according to the work of Deol (1973) and Hébert et al. (1985b). Thus, the common view has been that a critical, thyroxin-dependent period exists in mice and rats that extends from just before birth to roughly the middle to end of the second postnatal week, approximately coincident with the first sign of sound-elicited cochlear activity.

The specific aims of the current study were to assess the capacity of prenatal exogenous T_4 replacement to prevent auditory dysfunction, ascertained using the auditory brainstem response (ABR), and to determine the relative potency of T_4 replacement during key postnatal developmental periods in the *Tshr^{hyt}* homozygous mutant mouse.

MATERIALS AND METHODS

Study design

ABRs were recorded from *hyt/hyt* mice produced by male *hyt/hyt* × female *hyt/hyt* breeding pairs. This breeding strategy produced *hyt/hyt_(h)* offspring, according to the nomenclature of Sprenkle et al. (2001b), where the “h” subscript represents the hypothyroid condition of the dam from which the pups were derived; when offspring were derived from euthyroid, heterozygous dams, they are designated with an “e” subscript. Experimental subjects were treated with T_4 or saline placebo during specified prenatal and postnatal periods between conception (E0) and P28. Five separate groups were studied:

1. No T_4 —received placebo throughout the period of study;
2. T_4 : P0–P28—received T_4 during the postnatal period only (P0–P28);
3. T_4 : E0–E21—received T_4 during the prenatal period only; note that gestational period was not monitored for each litter and the E21 designation simply refers to the end of the prenatal period;
4. T_4 : E0–P14—received T_4 throughout the prenatal period and during the first 14 postnatal days;
5. T_4 : E0–P28—received T_4 throughout the prenatal period and throughout the postnatal period of study.

Nine to 12 mice were included in each group and subjects were drawn from 20 litters. Creighton University’s Animal Care and Use Committee approved the protocols employed in the care and use of animals used in this study.

Animal husbandry

Animals were bred and housed as described in Sprenkle et al. (2001a). Formulab Chow 5008 (Purina Mills, Inc., Richmond, IN, USA) was supplemented with thyroid powder prior to and between matings to enhance fertility. Supplementation was discontinued in female breeders at the time thyroxin pellets were implanted (see below). Both male and female progeny remained with the dam until the time of testing on P28.

T_4 replacement therapy

Prenatal treatment of animals *in utero* was achieved by administering thyroxin (T_4) to female breeders according to a standard protocol that calls for treatment to begin on P42, two weeks prior to mating. Animals were implanted subdermally with 60-day controlled-release pellets containing either placebo or L-thyroxin (Innovative Research of America, Toledo, OH, USA) using a standard trochar approach.

Postnatal T_4 or saline placebo was administered according to the plan described in Sprenkle et al. (2001a). Variable doses of L-thyroxin designed to mimic normal age-matched thyroxin titers were injected subcutaneously on days 0–5 (4 ng/g), 6–8 (5.8 ng/g), and 9–10 (9.1 ng/g) (Hébert et al. 1985b; Sprenkle et al. 2001b). Treatment was continued through either P14 or P28 according to a protocol requiring the administration of 14.0 ng/g on P11–P14 and alternate days between P16 and P28 (Dubois and Dussault 1977).

Serum T_4 determinations

Serum T_4 levels were determined in dams 3 and 50 days following pellet implantation and in experimental subjects on the day of testing, as described in Sprenkle et al. (2001a). Blood was collected from the tail vein and 5- μ L aliquots were assayed in duplicate by radioimmunoassay. The assay detection limit for T_4 was 0.01 μ g/dL.

Behavioral assessments

Daily notations were made to identify gross physical changes (e.g., body weights and ages associated with eye-opening and pinna-unfolding) and behavioral differences (circling tendency, righting reflex, and posture) among the groups. Behavioral evaluations were made on P21 and P28 as described in Sprenkle et al. (2001a).

ABR measurements

ABRs were assessed on P28 [the procedures used are described in detail in Sprenkle et al. (2001a)]. Briefly, subjects were anesthetized using chloral hydrate (480 mg/kg IP) and supplemented as needed (120 mg/kg IP). ABRs were recorded using subdermal electrodes positioned at the vertex (active), mastoid region (reference), and over the neck musculature (ground). Differentially recorded signals were amplified (100,000 $\times$), band-pass filtered (0.03–10 kHz), and digitized at a 20-kHz sampling rate over a 20-ms epoch that included a 5-ms baseline recorded prior to stimulus delivery. Each average consisted of 500 trials.

Digitally synthesized tone bursts (3 ms in duration with 1-ms on/off ramps) and clicks (60 μ s in duration) were alternated in polarity and delivered at a rate of 5/s under free-field conditions via a high-impedance piezoelectric tweeter positioned 10 cm above the vertex. Stimulus levels (in dB SPL) are referenced to 20 μ Pa. Thresholds were determined for clicks and for tone bursts at 20, 16, 8, 4, and 2 kHz. Response level series were acquired using both clicks and tone bursts (20 and 8 kHz) starting below threshold and extending to 90 dB SPL in 5-dB steps. Two responses were acquired at each level.

Data analysis

Analysis of variance (ANOVA) was used to evaluate differences in body weights and ages of eye-opening and pinna-raising among the experimental groups. Tukey tests were used for multiple comparisons. ABR thresholds, wave latencies, interpeak intervals, and amplitudes were measured as in Sprenkle et al. (2001a). Nonparametric statistics (Kruskal–Wallis test) were used for intergroup comparisons and differences were considered significant at $p < 0.01$, unless, otherwise stated.

RESULTS

Somatic and behavioral characteristics

Thyroxin serum levels in *hyt/hyt* dams implanted with controlled-release T_4 pellets were significantly ($p <$

FIG. 1. Serum T_4 levels in *Tshr* mice. Titers of serum T_4 in *hyt/hyt* dams were measured 3 and 50 days following placebo (saline) or thyroxin pellet implantation and are compared with levels measured in 28-day-old *hyt/hyt_e* and *+hyt_e* mice derived from euthyroid, heterozygous dams (from Sprenkle et al. 2001b). Values represent means and standard errors.

0.001) higher ($5.07 \pm 0.17 \mu\text{g/dL}$, $n = 12$) than in placebo-implanted dams ($0.59 \pm 0.07 \mu\text{g/dL}$, $n = 8$) at both 3 and 50 days following implantation (Fig. 1). Thus, all T_4 -treated dams were euthyroid throughout the period of pregnancy according to the commonly accepted criterion of $> 4.0 \mu\text{g}$ of T_4/dL of serum (Adams et al. 1989). Moreover, as shown in Figure 1, T_4 titers in placebo- and thyroxin-treated dams were comparable to values measured in untreated hypothyroid (*hyt/hyt_e*) and euthyroid (*+hyt_e*) mice derived from euthyroid, heterozygous dams, respectively (Sprenkle et al. 2001b). These results confirm that all experimental groups treated with thyroxin prenatally (i.e., the T_4 : E0–E21, T_4 : E0–P14, and T_4 : E0–P28 groups) were exposed to significantly higher levels of T_4 during prenatal life than groups born to untreated dams that were homozygous for the *hyt* allele (i.e., the No T_4 and T_4 : P0–P28 groups).

Serum thyroxin titers for *hyt/hyt* pups that were treated throughout postnatal development (circles) are compared with untreated pups (squares) in Figure 2. Note that the concentrations of T_4 in animals receiving replacement therapy mimic those of age-matched heterozygote controls (derived from euthyroid dams), whereas those receiving placebo mimic those of *hyt/hyt* pups derived from either heterozygous or homozygous mutant dams (Sprenkle et al. 2001b). Serum T_4 titers decreased rapidly when thyroxin treatment was discontinued on P10 and P14, although elevated levels were observed for 2–4 days following the last treatment.

Both groups actively receiving T_4 on P28 (T_4 : P0–

FIG. 2. Developmental changes in serum T_4 levels in placebo- and thyroxine-treated mice. The relationship between serum T_4 titers and age is shown for individual *hyt/hyt(h)* mice that were not treated with thyroxine (placebo controls; open squares), and those that were treated with T_4 at the indicated age ranges (see symbol key). Lines represent averages for each group. Data used for this figure were obtained from a separate set of animals than that used for ABR recordings (Sprenkle 1997). Asterisks represent serum titers for *+/hyt(e)* mice at P12, P15, P21, and P28 from Sprenkle et al. (2001b).

P28 and T_4 : E0–P28) were euthyroid based on assay findings at the time of ABR testing and T_4 titers were essentially the same (Table 1). Likewise, T_4 levels were unambiguously in the hypothyroid range at the time of testing when T_4 treatment was stopped on P14 or earlier.

All mice born to T_4 -treated dams had higher body weights ($p < 0.001$) than mice born to placebo-treated dams by P28, and mice treated throughout the study period (T_4 : E0–P28) weighed significantly more than animals whose treatment ended on P14 (T_4 : E0–P14) (Table 1). Body weight differences between animals treated with T_4 prenatally and those that were not were observed at all ages studied, and differences between

the groups increased with age (Sprenkle 1997). Pups treated after birth but not prenatally (T_4 : P0–P28) grew at the same rate as untreated animals (No T_4), underscoring the importance of prenatal thyroxine and the limited developmental advantage gained by exposing homozygotes to thyroxine during postnatal life alone.

Consistent with body weight growth findings, T_4 treatment affected the development of other notable somatic features like eye-opening and pinna-raising. Both of these indices of maturation occurred earlier ($p < 0.001$) in individuals treated through P14 or P28, relative to other groups studied in this investigation (Table 1). In addition, circling behavior was observed in a small percentage of animals that were not treated prenatally (i.e., 22% of the No T_4 group and 11% of the T_4 : P0–P28 group) and a small percentage of the group that was treated only prenatally (10% of the T_4 : E0–E21 group). Circling behavior was not observed in the 2 groups that were treated prenatally and postnatally through P14 or P28 (T_4 : E0–P14 and T_4 : E0–P28). Out of the entire study, only one animal responded abnormally to the tail-hanging test, and this individual also displayed circling behavior. Interestingly, this subject was treated for the full postnatal regimen but not prenatally.

General findings

As noted in Sprenkle et al. (2001b), *hyt/hyt* mice that are born to hypothyroid dams fail to develop normal auditory function. Consistent with that finding, all untreated animals studied here were remarkably abnormal on the day of testing (i.e., P28). This is clear when responses produced by high-level stimuli are compared for individuals that were hypothyroid (i.e., No T_4) and those that were euthyroid (i.e., T_4 :

TABLE 1

Somatic and behavioral characteristics

	No T_4	T_4 : P0–P28	T_4 : E0–E21	T_4 : E0–P14	T_4 : E0–P28
Serum T_4 ¹ (µg/dL)	0.34 ± 0.10	4.82 ± 0.34 ^a	0.38 ± 0.09	0.32 ± 0.08	5.13 ± 0.22 ^a
Birth weight ² (g)	1.23 ± 0.11 ^b	1.28 ± 0.19 ^b	1.49 ± 0.12	1.41 ± 0.14	1.44 ± 0.15
Weight at P28 ² (g)	9.2 ± 0.5 ^c	9.9 ± 0.4 ^c	11.7 ± 1.6 ^d	13.0 ± 0.8 ^d	16.5 ± 0.6 ^d
Eye-opening age ³	17.1 ± 1.2 ^e	17.2 ± 0.8 ^e	16.5 ± 0.8 ^e	15.2 ± 1.1	14.2 ± 1.1
Pinna-raising age ³	20.0 ± 1.1 ^e	20.0 ± 1.4 ^e	19.5 ± 1.4 ^e	17.4 ± 1.2	16.4 ± 1.4
Tail-hanging test	0/9	1/9	0/10	0/10	0/12
Circling behavior	2/9	1/9	1/10	0/10	0/12

Sample size for each measurement is indicated as the total number of animals tested for circling behavior under the appropriate column.

¹Mean ± SEM.

²Mean ± SD.

³Mean ± SD, in postnatal days.

^a $p < 0.001$ vs. the following groups: No T_4 , T_4 : E0–E21, and T_4 : E0–P14.

^b $p < 0.01$ vs. T_4 : E0–E21.

^c $p < 0.001$ vs. T_4 : E0–E21, T_4 : E0–P14, and T_4 : E0–P28.

^d $p < 0.001$ vs. all other groups.

^e $p < 0.001$ vs. T_4 : E0–P14 and T_4 : E0–P28.

FIG. 3. Representative examples of ABR waveforms. ABR records are shown for individual mice within each of the five experimental groups (A–E) in response to clicks (top rows), 20 kHz (middle rows), and 8 kHz (bottom rows). For A–D, ABR waveforms of 3 mice are shown, and in E, waveforms of 2 mice are shown. Stimuli were presented at 90 dB SPL. Two replicates are shown overlapping for each individual.

E0–P28) throughout the study period, as shown in Figures 3A and E. While approximately 20% of the untreated animals responded to 90 dB SPL tone bursts at 20 kHz, even fewer responded to 8 kHz, and clicks were completely ineffective at that level.

When T_4 treatment was limited to the postnatal period (T_4 : P0–P28), individuals were slightly more responsive than their untreated counterparts, while the difference between responses observed in pups treated only during the postnatal period (T_4 : P0–P28) and those treated throughout the pre- and postnatal period were obvious (Figs. 3B and E). Responses were highly variable among mice whose treatment ended at birth (T_4 : E0–E21), but all four waves were easily identified in most animals (Fig. 3C).

The influence of thyroxin treatment after P14 was also substantial, as indicated in the gross comparison of ABR waveforms associated with T_4 : E0–P14 and T_4 : E0–P28 groups (Figs. 3D and E). Although all animals responded at 90 dB SPL for the three stimulus conditions shown, responses recorded from individuals that were euthyroid throughout the study period were more robust and showed signs of greater maturity, as in reduced intragroup variability, shorter latencies, and larger amplitudes. In general, ABRs elicited from this group were completely normal, with all four major peaks occurring within the first 5 ms of the

response. As with ABRs from other mammals, wave splitting was relatively common at high stimulus levels for waves II, III, and IV. In particular, wave II often split into two peaks at levels ≥ 80 dB SPL and the appearance of a trailing shoulder on wave IV was common. Adjacent peaks occasionally merged, particularly between waves II and III, and this phenomenon occurred more frequently in response to tone bursts than in response to clicks. Overall, response waveforms appeared to exhibit normal level-dependent alterations, including decreased amplitudes and increased latencies as stimulus level was decreased (Fig. 4).

Incidence of response

The overall responsiveness of experimental groups studied as a part of this investigation was determined by plotting the percentage of animals within each group that responded to a specific stimulus as a function of level (Fig. 5). This analytical approach allows the straightforward comparison of responsiveness among the study groups and, consequently, the determination of the relative efficacy of thyroxin treatment during specified developmental stages. In such an analysis, a smooth, rapidly growing function that

FIG. 4. Level-dependent changes in ABR waveforms. ABR records from each experimental group (columns) are plotted in response to a range of levels for clicks (top rows), 20 kHz (middle rows), and 8 kHz (bottom rows). Each set of waveforms represents responses to stimuli starting at 90 dB SPL (upper trace of set) and decreasing in 10-dB steps (successively lower traces). Two replicates are shown overlapping at each level. Note the change in amplitude scale for the fully treated group (T₄: E0-P28) in the rightmost column.

reaches 100% at a relatively low stimulus level is characteristic of a highly responsive group whose thresholds are distributed normally over a narrow range of values. Response incidence curves with those characteristics were observed among animals that were treated with thyroxin throughout the full range of the study period (i.e., the T₄: E0-P28 category) (Fig. 5, diamonds). A clearly detectable portion (~10%) of that population responded to stimulus levels in the range of 10–15 dB SPL for frequencies that lie in the most sensitive part of the heterozygote's audiometric range (20 and 16 kHz), whereas clicks and 8-kHz tones had to be as high as 20–30 dB SPL to elicit responses in a small percentage of the population. For lower-frequency stimuli falling outside the most sensitive band of the audiometric range, ~10% of the group responded to 40–45 dB SPL for 4-kHz tones and 65–70 dB SPL for 2 kHz.

The only stimulus conditions producing responses in the untreated group (No T₄) were very high-level tone bursts. Threshold responses always exceeded 80 dB SPL and the percentage of untreated animals that responded to any acoustic stimulus never exceeded 50%.

Response incidence curves representing intermediate treatment groups, designated T₄: E0-P14, T₄: E0-E21, and T₄: P0-P28, indicated that the efficacy of thyroxin treatment was graded and dependent on

developmental stage. When thyroxin was administered throughout the combined prenatal period and the first 14 postnatal days, the overall responsiveness of the group was diminished relative to those treated throughout the entire study period as indicated by the rightward shift of sensitivity curves for all stimulus conditions. Unlike the smooth growth curves representing the T₄: E0-P28 group, growth curves were irregular, suggesting that the distribution of thresholds within those populations was broad. The level required to produce responses among the most sensitive members of this group was slightly higher (~10 dB for most conditions) than that observed in the group that was treated throughout the study period. However, as indicated by the extended dynamic range of representative population functions, many if not most individuals were notably less sensitive than the most sensitive representatives of the group, frequently by as much as 30 dB.

Animals treated during the prenatal period alone (the T₄: E0-E21 group) were more responsive than either untreated animals or individuals treated during the full postnatal period, but not prenatally (the T₄: P0-P28 group). The most sensitive animals responded in the range of 60–80 dB SPL above 4 kHz and incidence curves were generally smooth and steep, suggesting that thresholds are tightly and normally distributed among individuals in this group.

FIG. 5. Effects of thyroxin treatment on response incidence–stimulus level curves. The percentage of mice within each study group that responded to clicks and tone bursts is plotted as a function of stimulus level. The symbol key for all panels is located in the bottom-right panel. Nine to 12 mice were tested in each group.

This finding supports the view that prenatal exposure to T_4 is relatively more important to inner-ear development than postnatal exposure alone.

ABR thresholds

As expected based on findings from the analysis of response incidence curves, when detectable, the thresholds of untreated (No T_4) mice were very near the output limits of the sound delivery system (Fig. 6). Although somewhat more sensitive on average, thresholds of animals treated during the full postnatal period but not prenatally (the T_4 : P0–P28 group) were also very high, in the neighborhood of 90–100 dB SPL. While mice that were treated in the prenatal

period alone (T_4 : E0–E21) were more sensitive to acoustic stimulation than those treated in the postnatal period alone, the enhanced sensitivity did not achieve statistical significance relative to the untreated group for any stimulus condition. However, threshold improvement became significant relative to untreated mice (No T_4) when T_4 treatment was continued through at least P14 (T_4 : E0–P14), and fully treated mice (T_4 : E0–P28), who were comparable to individuals in the *hyt*/ $+$ _(e) category of Sprenkle et al. (2001b), had the lowest thresholds of all five groups at all frequencies tested. These improvements were significant with respect to all groups except those who received T_4 through P14.

As shown in the form of response incidence curves

FIG. 6. Effects of thyroxin treatment on frequency–threshold curves. Average ABR thresholds of each experimental group (see symbol key) are plotted as a function of stimulus frequency. Thresholds for click stimuli are also shown. Upward-pointing arrows denote instances in which one or more mice exhibited no measurable response at the highest stimulus level used. Error bars represent the SEM of 9–12 mice tested per group. Dashed line represents thresholds of 28-day-old heterozygotes derived from euthyroid dams (+/*hyt*_(a)) reported in Sprenkle (2001b). Letter designations next to symbols represent statistically significant differences ($p < 0.01$) between the indicated group and the following groups: “a,” versus No T₄ and versus T₄: P0–P28; “b,” versus No T₄, versus T₄: P0–P28, and versus T₄: E0–E21.

previously, the discovery that prenatal thyroxin has a nonlinear cumulative influence on subsequent development is clear when threshold–frequency plots for animals treated throughout the postnatal period alone (T₄: P0–P28) are compared with threshold–frequency plots representing individuals that were treated during the prenatal period only (T₄: E0–E21). It is clear that postnatal exposure to thyroxin is essentially ineffective when an individual’s prenatal history is one of hypothyroidism.

Input–output relationships

Input–output (I/O) functions were analyzed with respect to absolute latencies, interpeak intervals, and amplitudes for all five groups studied. Because of the high thresholds that are characteristic of groups that did not receive prenatal T₄, responses from No T₄ and T₄: P0–P28 groups were often unobtainable, or, at best, individuals from these groups responded to only the highest stimulus levels studied. For this reason, analysis of input–output characteristics was limited to the three groups that were treated during the prenatal period. When available, however, mean responses of the No T₄ and T₄: P0–P28 groups are shown for comparison with other groups (i.e., Figs. 8, 10, 11, and 13). As shown in relevant figures, response latencies were greatly prolonged and amplitudes greatly reduced among individuals that were not exposed to prenatal T₄ relative to individuals treated during prenatal life.

ABR wave latencies. The overall form of latency–intensity curves representing the groups studied here was consistent with the well-established principle that response latency decreases as stimulus level increases (Fig. 7). Response variability was relatively low and input–output (I/O) curves were tightly distributed when animals were treated with thyroxin over the entire study period (i.e., the T₄: E0–P28 condition). I/O curve slopes and dynamic ranges were similar among members of this group, regardless of wave number (Fig. 7, right column).

On the other hand, response latencies were widely distributed and notably prolonged among a large portion of individuals who received thyroxin during the prenatal period only (T₄: E0–E21 group) (Fig. 7, left-most column). Dynamic range was notably small among representatives of this group and the slopes of individual curves were similar. When latency–intensity curves from animals that had been treated throughout prenatal life *plus* the first 14 postnatal days (T₄: E0–P14) were compared with this group, a subpopulation responded with normal wave I latencies to high-level stimulation (i.e., > 60 dB SPL), although response latencies from the overall population were highly variable (Fig. 7, middle column), as in the case of the T₄: E0–E21 group.

The cumulative advantage of administering T₄ throughout development, and the essential nature of prenatal exposure to the hormone for normal auditory development, is graphically depicted in the form of average latency–level curves for responses to clicks and tone bursts of 20 and 8 kHz in Figure 8. When thyroxin was withheld during the prenatal period (squares and circles), although mean latencies generally decreased with level, response latencies were greatly prolonged regardless of condition or wave number. When considering those experimental groups that were exposed to prenatal thyroxin (triangles and diamonds), mean latencies also decreased with level, although latencies rarely achieved normal values when thyroxin was discontinued on or before P14. It is interesting that the extent of recovery, here defined in relation to normal age-matched values, was dependent on the degree of thyroxin exposure (see below). Notably, prenatal exposure to thyroxin alone (upward-pointing triangles) produced more recovery of function than thyroxin administered throughout the entire first postnatal month alone (circles). The occasional irregularity observed in average latency–level curves for the T₄: E0–P14 group (downward-pointing triangles in Fig. 8) is indicative of the bimodal character of latency–level functions comprising this group.

Although more evident in some cases than others, latency was inversely related to the duration of thyroxin exposure (Fig. 9, upper panels), as was the steepness

FIG. 7. Latency-level functions of thyroxin-treated mice. Click-evoked latencies for ABR waves I-IV (top to bottom rows) are plotted as a function of stimulus level for three of the experimental groups (columns). Each curve represents the responses of one individual.

FIG. 8. Effects of thyroxin treatment on latency-level functions. Mean latencies of ABR waves I-IV (top to bottom rows) for each responsive group are plotted as a function of stimulus level for clicks (left column), 20 kHz (middle column), and 8 kHz (right column). Symbol key for all panels is shown in the lower-middle panel. Means are shown for only two or more values at a given stimulus condition, except for the No T_4 and T_4 : P0-P28 groups for which means from all responses are included.

of latency-level curve slopes among animals treated with thyroxin during the prenatal period (Fig. 9, lower panels), regardless of stimulus conditions. Latency differences between those homozygotes that were treated

with T_4 during prenatal life only (T_4 : E0-E21) and those treated throughout the period of study (T_4 : E0-P28) were significant in the case of wave I for clicks and tone bursts presented at 90 dB SPL and, although

FIG. 9. Influence of thyroxine treatment duration on latencies and slopes of latency-level curves. Mean latencies at 90 dB SPL (top row) and mean slopes of latency-level curves (bottom row) are plotted for ABR waves I through IV in response to clicks (left column), 20 kHz (middle column), and 8 kHz (right column), as a function of thyroxine (T_4) treatment duration. Values plotted at 9 days represent animals that were treated prenatally only (T_4 : E0–E21); 9 days is based on the age range from the time that thyroid hormone receptors are first detected in the inner ear (Bradley et al. 1994) to the time of birth (21 days gestation). Values at 23 days represent treatment during the 9 days *in utero* to P14 (T_4 : E0–P14 group), and the values at 37 days represent an additional 14 days of treatment (T_4 : E0–P28 group). Slopes of latency-level curves were computed by normalizing latencies to the value measured at 90 dB SPL (subtracting that value and adding 1), converting to logarithmic values, and then performing a least-squares linear regression to the data.

not achieving significance, other waves followed the same pattern. There were statistically significant differences ($p < 0.01$) in slopes of latency-level curves between animals treated for 9 days (T_4 : E0–E21 group) and those treated throughout the study period (T_4 : E0–P28) for waves I–IV in response to tone bursts and for waves III and IV in response to clicks. In general, the overall picture is similar to that observed in developing $+ / hyt$ animals (Sprenkle et al. 2001b).

The extent to which threshold differences contributed to latency prolongation in affected animals was estimated by comparing the form of latency-intensity curves expressed in sensation level terms, among groups, as shown in Figure 10 for the 20-kHz condition. Although latency differences near threshold are apparent, with latencies of all four waves prolonged in the T_4 : E0–E21 group relative to the T_4 : E0–P28 group, differences were only significant for wave I, and these differences disappeared for all waves when intensity was raised to a level approximately 30 dB above threshold. Similar trends were observed for click-evoked and 8-kHz-evoked responses, indicating that threshold was only one of the factors affecting latency prolongation among animals that were treated for only a portion of the study period.

ABR interpeak intervals. Interpeak intervals (I–II, II–III, and III–IV) and central conduction times (I–IV interval) measured as a function of stimulus level did

not differ among the three groups of mice that received T_4 throughout prenatal life. However, greatly prolonged mean intervals at 90 dB SPL were observed in untreated animals (No T_4) and intermediate intervals were measured in those receiving thyroxine replacement only during the postnatal period (T_4 : P0–P28) (Fig. 11).

ABR wave amplitudes. The relative importance of thyroxine as a maturational agent affecting auditory system development, and peripheral function in particular, in the latter half of the first month is apparent when even casually inspecting click-evoked amplitude-intensity plots in Figure 12. While amplitudes are highly variable, it is relatively clear that responses were generally more robust in animals treated throughout the study period relative to either partial replacement condition, particularly for waves I and IV. It is equally clear that threshold improvement is the singular most dramatic parameter change associated with thyroxine treatment during the last half of the first postnatal month. Wave II was strikingly nonmonotonic in fully treated animals, but the nonmonotonicity was absent from the records of partially treated animals, presumably a reflection of high thresholds in those groups. Similar observations were made for amplitude-intensity functions generated from responses to 20 and 8 kHz.

Although variable from condition to condition, the

FIG. 10. Latency-sensation level functions in placebo- and thyroxin-treated mice. Mean latencies of ABR waves I-IV for each experimental group are plotted as a function of sensation level in response to 20-kHz tone bursts. (Symbol key for all panels is shown in the upper-right panel.) Sensation level was computed by subtracting the ABR threshold of each individual from the absolute sound pressure level. Means are shown for only two or more values at a given stimulus condition, except for the No T_4 and T_4 : P0-P28 groups, for which means from all responses are included.

FIG. 11. Comparison of interpeak interval-vs-level curves in placebo- and thyroxin-treated mice. Mean interpeak intervals between the first four ABR waves (I-II, II-III, and III-IV, top three rows) and central conduction time (wave I-IV interval, bottom row) for each experimental group are plotted as a function of stimulus level for clicks (left column), 20 kHz (middle column), and 8 kHz (right column). (Symbol key for all panels is located in the lower-right panel.) Means are shown for only two or more values at a given stimulus condition, except for the No T_4 and T_4 : P0-P28 groups for which means from all responses are included.

cumulative effect of treatment duration is relatively clear in a composite plot of average amplitude-level curves representing responses to clicks, 20 kHz, and 8 kHz (Fig. 13). Again, the importance of prenatal

thyroxin is apparent in that members of both untreated groups (No T_4 and T_4 : P0-P28) were either unresponsive or barely responsive to clicks or tone bursts, even at the highest stimulus levels used in the

FIG. 12. Amplitude-level functions of thyroxin-treated mice. Click-evoked amplitudes for ABR waves I-IV (top to bottom rows) are plotted as a function of stimulus level for three of the experimental groups (columns). Each curve represents the responses of one individual.

FIG. 13. Effects of thyroxin treatment on amplitude-level functions. Mean amplitudes of ABR waves I-IV (top to bottom rows) for each responsive group are plotted as a function of stimulus level for clicks (left column), 20 kHz (middle column), and 8 kHz (right column). (Symbol key for all panels is shown in the lower-right panel.) Means are shown for only two or more values at a given stimulus condition, except for the No T_4 and T_4 : P0-P28 groups for which means from all responses are included.

study. The other remarkable finding revealed in average amplitude plots, like those shown here, is the relative importance of thyroxin beyond the second postnatal week. This is illustrated most dramatically

for responses to clicks (left column), although the finding holds for the other stimulus conditions as well.

At 90 dB SPL the effect of postnatal treatment duration on response amplitude was most notable for wave

I. The average amplitude of wave I in animals treated throughout the study period was more than twice that measured in mice whose treatment was discontinued on P14 (T_4 : E0–P14) ($p < 0.001$) and four times that measured for the prenatal group for most stimulus conditions.

DISCUSSION

The principal finding of this study is that peripheral auditory dysfunction can be prevented in the genetically hypothyroid *hyt/hyt* mouse by the exogenous administration of thyroxin from the time of conception through the end of the first postnatal month. It confirms the earlier suggestion of Sprenkle et al. (2001a) that normal hearing in *hyt* mice requires that thyroxin be delivered beyond the onset of hearing and extends that observation to affirm that the hormone is required throughout the period of cochlear development. A second major finding of this study is that the postnatal administration of thyroxin to prenatally hypothyroid homozygotes is largely ineffective.

T_4 deficiency is the major cause of auditory abnormalities in homozygotes

In a companion study (Sprenkle et al. 2001a), we confirmed the observation of Deol (1973) that auditory dysfunction in hypothyroid mice born to euthyroid dams could be partially blocked by treating animals with thyroxin during the first 10 postnatal days of life. The rationale underlying that study was based on the historical perspective that a thyroxin-dependent critical period of development occurs in neonates prior to the onset of hearing in the mouse (Deol 1973). Deol observed that mice treated with thyroxin after postnatal day 0 develop essentially normal cochlear anatomy and suggested that the sensitivity of the auditory system to thyroid hormones declines following the attainment of cochlear function, which in the mouse occurs from P9 to P14 (Alford and Ruben 1963; Kikuchi and Hilding 1965; Mikaelian and Ruben 1965). However, two separate sets of data collected in the series of experiments reported here support the notion that sensitivity to thyroid hormone extends beyond the middle of the second postnatal week in the *hyt* mouse.

First, and perhaps most important, although exogenous thyroxin protected homozygous pups born to heterozygous dams from hypothyroid-induced dysfunction when administered between birth and P10, the protection was at best partial (Sprenkle et al. 2001a). That finding naturally led to the current study, and, based on results shown here, we are able to

unequivocally state that thyroxin is an essential factor in auditory development beyond P14.

This finding should not be particularly surprising in light of the fact that notable cochlear development continues well beyond the age that airborne sounds first elicit detectable responses from the auditory system (Kraus and Aulbach–Kraus 1981; Shnerson et al. 1982). It is entirely reasonable to assume that thyroid hormones may be required for these late-stage events in cochlear differentiation. Given the fact that the organ of Corti is essentially adultlike after P14, one is drawn to consider the possibility that the efferent olivocochlear system may be especially important as a maturational factor during this late period since it is one of the last features of the system to organize (Lenoir et al. 1980; Shnerson et al. 1982; Simmons et al. 1998).

Prenatal T_4 contributes significantly to hearing development

Traditionally it was held that thyroid hormones do not play a role in the development of audition during the prenatal period that preceded autonomous thyroxin secretion. Until recently, convincing evidence that maternal hormones are transported across the placenta or that fetal thyroid hormone receptors are expressed early enough to effect development was unavailable. This was true even though the devastating consequences of maternal iodine deficiency on fetal brain development were well documented (Delange 1991). With the discovery that significant amounts of maternal thyroxin are taken up by the fetal brain (Porterfield and Hendrich 1992), combined with the discovery that thyroid hormone receptors are expressed in both the brain (Pérez–Castillo et al. 1985) and cochlea (Bradley et al. 1994) well before the onset of autonomous fetal secretion, the issue is being revisited.

The findings reported here add to a body of evidence supporting the view that maternal thyroid hormones play an important role during development of the central and peripheral auditory systems, particularly when fetal thyroid hormone production is impaired. But what about earlier stages of development? The thyroid hormone receptors $TR\beta 1$ and $TR\beta 2$ are expressed very early in inner-ear organogenesis and explicitly in the anlagen of the cochlea, while $TR\alpha$ is expressed throughout the statoacoustic end organ (Bradley et al. 1994). This early expression, an event that occurs long before the system is responsive to sound, clearly points to the notion that inner-ear tissues contain thyroxin-responsive genes that are responsible for morphogenesis. Interestingly, however, cochlear malformations, including innervation patterns, are not observed in mice with null mutations

in the gene encoding TR β , even though affected animals are hearing-impaired (Forrest et al. 1996). Based on that observation, Forrest et al. suggests that the β form of the T₃ receptor controls functional maturation of the inner ear and the α form affects morphogenesis. By functional maturation, these authors presumably refer to thyroxine's capacity to regulate protein expression and thereby affect sensory mechanisms associated with mechanoelectrical transduction. However, auditory brainstem response thresholds are normal in mice with null mutations associated with TR α and TR β 2 (Rüsch et al. 1998; Abel et al. 1999), suggesting that cochlear development is not dependent on the activation of those signaling channels and raising the question, precisely what inner-ear tissues are effected by thyroid hormone? While precise T₃ targets in the embryonic ear are unknown, their identification will clearly promote a more comprehensive understanding of the mechanism(s) whereby thyroid hormones regulate auditory gene expression. The availability of the *hyt* mouse may facilitate that effort.

ACKNOWLEDGMENTS

This project was supported by grants from the Deafness Research Foundation and the NIH (DC01007, DC00215, DC00982).

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