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Hearing loss and retarded cochlear development in mice lacking type 2 iodothyronine deiodinase

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The later stages of cochlear differentiation and the developmental onset of hearing require thyroid hormone. Although thyroid hormone receptors (TRs) are a prerequisite for this process, it is likely that other factors modify TR activity during cochlear development. The mouse cochlea expresses type 2 deiodinase (D2), an enzyme that converts thyroxine, the main form of thyroid hormone in the circulation, into 3,5,3'-triiodothyronine (T3) the major ligand for TRs. Here, we show that D2-deficient mice have circulating thyroid hormone levels that would normally be adequate to allow hearing to develop but they exhibit an auditory phenotype similar to that caused by systemic hypothyroidism or TR deletions. D2-deficient mice have defective auditory function, retarded differentiation of the cochlear inner sulcus and sensory epithelium, and deformity of the tectorial membrane. The similarity of this phenotype to that caused by TR deletions suggests that D2 controls the T3 signal that activates TRs in the cochlea. Thus, D2 is essential for hearing, and the results suggest that this hormone-activating enzyme confers on the cochlea the ability to stimulate its own T3 response at a critical developmental period.

deafness | thyroid hormone receptor

Thyroid hormone regulates many developmental processes that vary in their tissue specificity and the stage at which they occur. An important question therefore concerns the means by which this systemic hormonal signal is able to elicit its specific actions in a given tissue at the appropriate time. Hearing is one of the most sensitive functions controlled by thyroid hormone, and early-onset hypothyroidism or iodine deficiency are known causes of deafness in humans and rodent model species (1–5). Thyroid hormone is required for the timely coordination of a complex set of differentiation events in the maturing cochlea. In rodents, the cochlea is largely formed by birth, and it then proceeds through a period of differentiation during which auditory function initiates around postnatal day 13 (P13) and matures by \approx P20 (6, 7). Hypothyroidism during this period retards the differentiation of the cochlea, including the epithelium that contains the sensory hair cells (1, 3, 8, 9). The mechanisms that prompt the progression of these developmental events are poorly understood.

Thyroid hormone receptors TR β and TR α 1 are ligand-regulated transcription factors and are expressed in the sensory epithelium and other tissues in the rodent cochlea from mid-gestation into the postnatal period (10, 11). TR β -deficient mice display auditory deficits with a subset of hypothyroid-like abnormalities in the cochlea (12, 13). TR α 1 is individually dispensable for hearing, whereas deletion of both TR β and TR α 1 produces exacerbated defects that resemble those caused by hypothyroidism (13–15). Some human TR β mutations are associated with hearing loss (16–18). Although TRs are necessary mediators of thyroid hormone signaling, it is unclear which other factors influence TR activity during cochlear development.

Thyroid hormone may be metabolically activated or inactivated by iodothyronine deiodinases (19–21). Thyroxine (T4), the

main product released by the thyroid gland into the circulation is converted by outer ring 5'-deiodination into 3,5,3'-triiodothyronine (T3) the principal ligand of the TR. This conversion is mediated by deiodinase type 2 (D2) and deiodinase type 1 (D1) encoded by *Dio2* and *Dio1*, respectively. Deiodinase type 3 (D3) depletes tissues of active hormone by inner ring deiodination of T4 and T3. The differential expression of D1, D2, and D3 in some tissues (22–26) raises the possibility that deiodinases play specific developmental roles. Previously, we identified prominent expression of D2 in the early postnatal mouse cochlea, suggesting a role for D2 in the development of hearing (27). D2 is expressed in the cochlear capsule and connective tissues that surround the internal sensory tissues. Here, we report an essential role for D2 in the development of the auditory system.

Methods

Mouse Strains. The targeted deletion of *Dio2* was carried on a mixed background of 129/SvJ \times C57BL/6 strains, as described (28). The mutation was backcrossed for two further generations onto the C57BL/6J strain (The Jackson Laboratory, no. 000664) that has minimal hearing loss before 6 months of age (13, 29). Genotyping was performed on genomic DNA by PCR with primers specific for *neo* in the *Dio2*^{-/-} mutant allele and for a region of intron 1 and exon 2 specific for the WT gene (GenBank accession no. NT_039552). Primer sequences were: *neo* sense and antisense, GTGTTCCGGCTGTACGCGC and GTCCTGAT-AGCGGTCCGCCA, respectively; and *Dio2* sense and antisense, GACCATCCTTTATATTGCCTGACATG and GGCG-GAAGGCTGGCAGTTGCC, respectively. *Dio2*^{-/-} and control littermates were derived from crosses of *Dio2*^{+/-} parents or *Dio2*^{-/-} male and *Dio2*^{+/-} females. Nursing dams or pregnant dams at 14.5–17.5 days postcoitum were treated with 0.3 or 1.0 μ g/ml L-triiodothyronine (Sigma) in drinking water. Animal studies were conducted under approved protocols at Mount Sinai School of Medicine and Dartmouth Medical School. Statistical analyses were performed by using the Student *t* test.

Auditory-Evoked Brainstem Response (ABR). The ABR was measured by using a SmartEP system (Intelligent Hearing Systems, Miami) as described (13, 29). Mice were anesthetized with avertin (0.25 mg/g of body weight) for the short duration of the test and on revival were returned to their cages. Stimuli were presented at a rate of 25/s and averaged across 512 sweeps. Responses were bandpass filtered <100 and >3,000 Hz and amplified 100,000-fold. A threshold was defined as the minimal

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Abbreviations: D1, deiodinase type 1; D2, deiodinase type 2; D3, deiodinase type 3; TR, thyroid hormone receptor; T3, triiodothyronine; T4, thyroxine; ABR, auditory-evoked brainstem response; TM, tectorial membrane; P(n), postnatal day n; SPL, sound pressure level.

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stimulus level that gave a recognizable waveform on a normalized scale. For presentation in figures, a fixed scale is used.

Histology, Immunohistochemistry, and Electron Microscopy. Cochleae were fixed in 3% glutaraldehyde/2% paraformaldehyde, decalcified, embedded in methacrylate, sectioned at 3–5 μm , and stained with thionin, as described (13). Groups of four to six cochleae from three mice were analyzed. Cryosections made from paraformaldehyde-fixed tissues were immunostained with antibodies against α - and β -tectorins by using detection with FITC-conjugated secondary antibodies (30). Tectorial membrane (TM) ultrastructure was examined in ultrathin sections by transmission electron microscopy, as described (13), in WT and *Dio2*^{-/-} mice ($n = 3$) at 1 month of age. Prior analysis of ABR thresholds confirmed the auditory phenotype of these mice: WT, 42 \pm 2 dB sound pressure level (SPL); *Dio2*^{-/-} mice, 73 \pm 3 dB SPL for a click stimulus.

Deiodination Assays and Hormone RIAs. For each genotype, three pools of cochleae each from \approx 20 pups at P8 were collected on dry ice before assaying D2 and D3 activity, as described (28). Cochleae were homogenized in assay buffer containing 0.25 mM sucrose, 20 mM Tris-HCl, pH 7.6, with 5 mM DTT. For D2 assays, the mixture (total volume 50 μl) contained 80–100 μg of tissue protein and 1.2 mM EDTA. Substrate was 1.0 nM of [¹²⁵I]reverse T3 or [¹²⁵I]T4, and cofactor was 20 mM DTT. Reactions were incubated for 1 h at 37°C in the presence or absence of 1 mM 6-*n*-propyl-2-thiouracil, a D1 inhibitor, and the [¹²⁵I]I⁻ generated was detected after separation by ion exchange chromatography on Bio-Rad AG 50WXG (H⁺) resin. For D3 assays, the mixture did not contain EDTA, substrate was 1.0 nM [¹²⁵I]T3, and cofactor was 50 mM DTT; products were separated for analysis by paper chromatography. [¹²⁵I]reverse T3, [¹²⁵I]T4, and [¹²⁵I]T3 (specific activities \approx 1,000 $\mu\text{Ci}/\mu\text{g}$) were obtained from Perkin-Elmer and purified by chromatography using Sephadex LH-20 (Sigma) before use. Total T4 and total T3 levels were determined by RIA using Coat-A-Count reagents (Diagnostic Systems Laboratories, Webster, TX) (28) or a chemiluminescent assay for T4 on an Advia Centaur instrument (Bayer HealthCare, Tarrytown, NY) and a fluorometric assay for T3 on an Abbott AxSYM instrument. Coefficients of variation were 6–10% for T4 and 5–13% for T3.

Northern Blot Analysis. Total RNA samples from pools of cochleae from P8 pups (\approx 30 per genotype) were analyzed with a glyceraldehyde-3-phosphate dehydrogenase probe and a 1.4-kb mouse D2 cDNA probe (B1), extending from the fifth codon to 561 bp downstream of the stop codon in the 3' untranslated sequence, as described (27).

Results

Auditory Deficits in *Dio2*^{-/-} Mice. *Dio2*^{-/-} mice are a suitable model for the analysis of auditory function as they lack gross developmental defects (28, 31). The deletion in the major coding exon of *Dio2* (28) allowed mice to be genotyped by PCR, as shown in Fig. 1A. Northern blot analysis detected a 7.0-kb D2 mRNA in cochlear samples from WT and *Dio2*^{+/-} pups but not from *Dio2*^{-/-} pups (Fig. 1B). Normally, cochlear D2 activity rises during the first postnatal week and is abundant at P8 (27). Cochlear homogenates from *Dio2*^{-/-} pups at P8 lacked 5'-deiodinase activity, assayed with T4 or reverse T3 substrates (Fig. 1C). Activity was reduced by \approx 50% in *Dio2*^{+/-} mice. The 5'-deiodinase activity displayed by WT and *Dio2*^{+/-} samples was attributable to D2 because it was not significantly different in the presence or absence of propylthiouracil, an inhibitor of D1. Inner ring deiodination by D3 was detected at similar low levels in cochlear samples from WT, *Dio2*^{+/-}, and *Dio2*^{-/-} pups at P8.

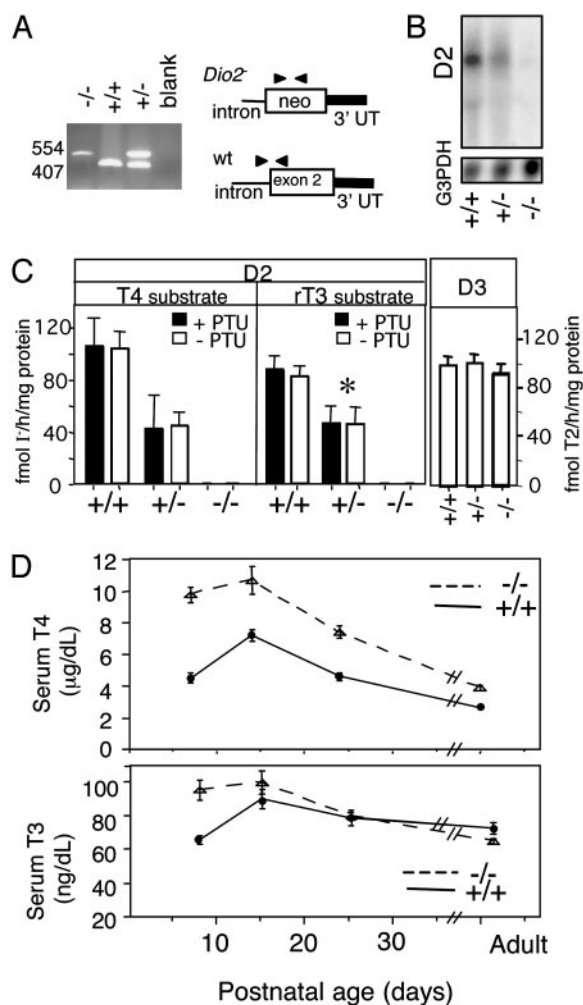


Fig. 1. Inactivation of cochlear D2 in *Dio2*^{-/-} mice. (A) *Dio2*^{-/-}, *Dio2*^{+/-}, and WT genotypes determined by PCR, based on the deletion in coding exon 2 in the mutant allele. Origins of primers specific for the mutant (*neo*) and WT alleles are indicated by arrowheads; sizes of products are indicated on the left (in bp). (B) Northern blot analysis of cochlear total RNA (15 μg per lane) from P8 pups detected a major 7-kb D2 band only in *Dio2*^{+/-} and *Dio2*^{-/-} mice. A glyceraldehyde 3-phosphate dehydrogenase (G3PDH) control probe was used on the same blot. (C) D2 5'-deiodinase activity in cochlear homogenates from P8 mice assayed with T4 or reverse T3 substrates. Activity was undetectable in *Dio2*^{-/-} samples, and in *Dio2*^{+/-} mice it was \approx 50% below WT levels (*, $P < 0.05$). Results were similar in the presence or absence of propylthiouracil (PTU), an inhibitor of D1. D3 activity was detected at similar, low levels in all genotypes. (D) Serum levels of total T4 and total T3 during development in *Dio2*^{-/-} and WT mice. In *Dio2*^{-/-} mice, T4 was somewhat elevated and T3 was normal or slightly elevated.

Thus, no compensatory changes in D1 or D3 were evident in the cochlea in *Dio2*^{-/-} mice.

To rule out systemic hypothyroidism in development as a cause of any deafness, thyroid hormone levels were determined in serum of WT and *Dio2*^{-/-} mice between P8 and P25 (Fig. 1D). *Dio2*^{-/-} pups displayed moderately elevated T3 and T4 levels, whereas *Dio2*^{-/-} adults, as reported previously, had slight T4 increases caused by a mild dysfunction of the pituitary–thyroid axis (28). The degree of elevation of T4 and T3 was greater in younger *Dio2*^{-/-} pups but moderated by P16. Thus, no general deficiency of thyroid hormones occurred in *Dio2*^{-/-} mice. Hypothyroidism presents a known risk to hearing but a modest thyroid hormone increase would not cause overt deafness (12, 32) (and see Fig. 5).

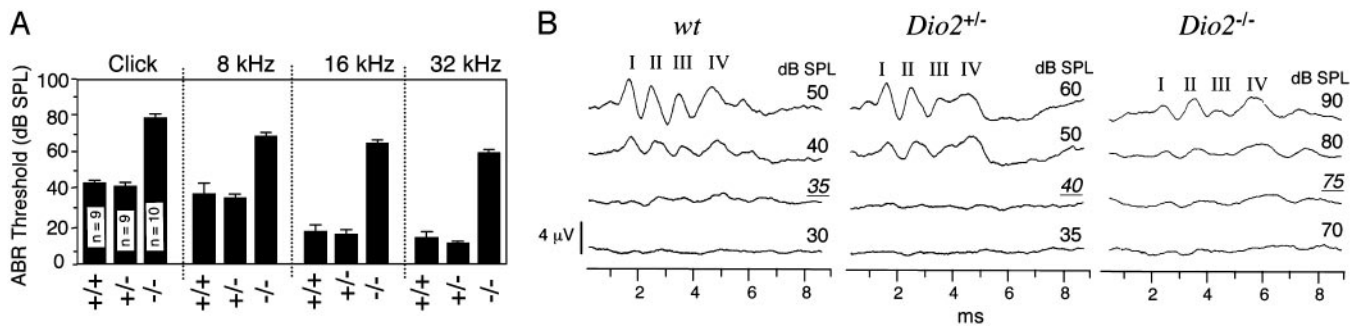


Fig. 2. Defective auditory function in *Dio2*^{-/-} mice. (A) Mean ABR thresholds \pm SEM in 1-month-old mice. *Dio2*^{+/+} and *Dio2*^{+/-} mice had normal thresholds for a click stimulus (a broadband of frequencies from 2 to 20 kHz) and pure tones of 8, 16, and 32 kHz. *Dio2*^{-/-} mice had elevated thresholds for all stimuli ($P < 0.001$). (B) Representative ABR waveforms for a click stimulus, shown on a 4- μ V fixed scale. Thresholds are underlined. Normally, four main peaks (marked I–IV) were detected within 5 ms of stimulation (a fifth peak is often not discernible in mice). In *Dio2*^{-/-} mice, waveforms were poorly defined with reduced amplitude and prolonged latency (assignment of specific peak numbers was precluded in some *Dio2*^{-/-} mice because of this poor definition).

To assess hearing, the ABR was measured in WT, *Dio2*^{+/-}, and *Dio2*^{-/-} littermates. Responses were markedly impaired in all *Dio2*^{-/-} mice and could be evoked only with a significantly increased SPL (Fig. 2A). In 1-month-old *Dio2*^{-/-} mice, the mean threshold for a click stimulus was 79 ± 2 dB SPL, an increase of 40 dB above normal (WT, 39 ± 2 dB SPL; $P < 0.001$), indicating a severe, although not total, impairment of auditory function. ABR waveforms in mice typically display four to five peaks with the first two to three peaks representing responses from the cochlea and proximal brainstem centers (29, 33). In *Dio2*^{-/-} mice, all peaks were poorly defined and had prolonged latency (Fig. 2B). Using a click stimulus at 90 dB SPL, mean latencies to peak IV in WT, *Dio2*^{+/-}, and *Dio2*^{-/-} mice were 4.06 ± 0.21 , 4.44 ± 0.05 , and 5.75 ± 0.19 ms, respectively ($n = 6$ – 8 ; $P < 0.001$ for *Dio2*^{-/-} vs. WT or *Dio2*^{+/-}). The ABR also showed elevated thresholds and poorly defined waveforms in *Dio2*^{-/-} mice for 8-, 16-, and 32-kHz stimuli that span the sensitive hearing range of mice. Similar threshold increases were recorded in 3-month-old *Dio2*^{-/-} mice, indicating a permanent defect (for a click stimulus; thresholds were 81 ± 1 and 42 ± 3 dB SPL, for *Dio2*^{-/-} and WT mice, respectively; $P < 0.01$; $n = 8$ *Dio2*^{-/-}, $n = 4$ WT). The auditory defects in *Dio2*^{-/-} mice were similar to or slightly worse than those in TR β -deficient mice (12, 13).

Abnormal Cochlear Morphology in *Dio2*^{-/-} Mice. Histological analysis in *Dio2*^{-/-} mice revealed retarded differentiation of the cochlear inner sulcus and sensory epithelium and deformity of the TM, a secreted extracellular matrix that is necessary for the response of the mechanosensitive hair cells to sound stimulation (30). The defects detected in *Dio2*^{-/-} mice resided in known thyroid hormone-sensitive tissues in the cochlear interior rather than in the surrounding connective tissues that normally express D2 (i.e., the cochlear capsule, the septal divisions between the turns of the cochlea, and the modiolus) (27).

Normally at P8, the sensory epithelium forms three distinct rows of outer hair cells and one row of inner hair cells separated by the tunnel of Corti, whereas the inner sulcus epithelium is partly retracted below the TM (Fig. 3A). In the adult, the TM extends fully across the cavity of the inner sulcus to contact the stereociliary bundles of the hair cells (Fig. 3D). In *Dio2*^{-/-} pups at P8, the sensory epithelium was poorly differentiated, the tunnel of Corti was unopened, and the inner sulcus was only at an early stage of formation (Fig. 3B). The inner sulcus epithelium was thicker with taller columnar cells than in WT pups. The TM was enlarged and attached for most of its length to the underlying epithelium. Occasionally, the TM was thinner and the inner sulcus was partly developed, suggesting variable penetrance for part of the phenotype (Fig. 3C). However, all *Dio2*^{-/-}

pups showed retarded differentiation of the sensory epithelium. The TM enlargement was evident as early as P4 but other morphological changes were not observed before P4 in *Dio2*^{-/-} mice.

In adult *Dio2*^{-/-} mice, the differentiation of many features of the sensory epithelium and the opening of the tunnel of Corti had progressed to resemble the WT cochlea. The TM, however, remained variably malformed (Fig. 3E). The TM deformity was

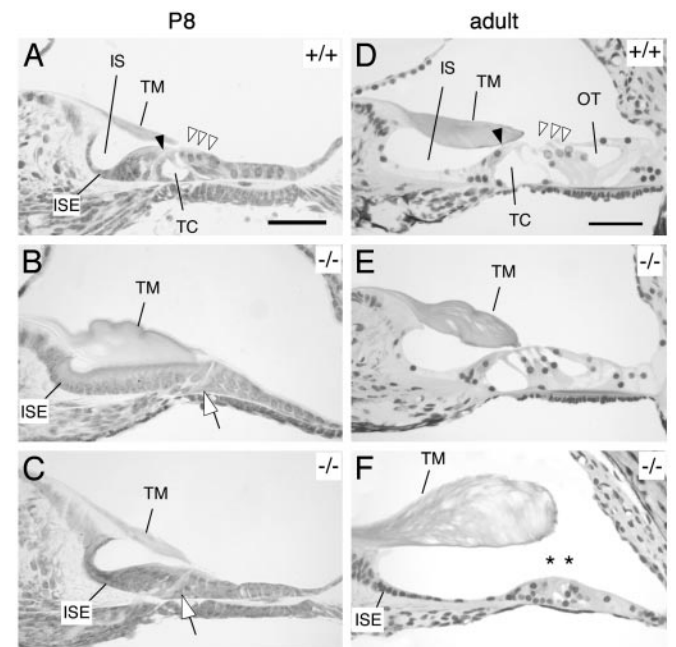


Fig. 3. Cochlear morphology in *Dio2*^{-/-} mice. (A–C) P8 pups. (A) In the WT, the tunnel of Corti (TC) has opened between the pillar cells adjacent to the inner (filled arrowhead) and outer (three open arrowheads) hair cells. The TM is thin and extends over the inner sulcus (IS). (B) In this *Dio2*^{-/-} pup, the TM is enlarged and attached to the underlying epithelium; the inner sulcus epithelium (ISE) is thick; the sensory epithelium, containing the hair cells, is poorly differentiated; and the TC is absent (arrow). (C) A *Dio2*^{-/-} pup, in which the TM is more normally shaped, but differentiation of the ISE and sensory epithelium is still retarded and the TC is absent (arrow). (D–F) Adults. (D) A WT cochlea, with mature formation of the TC, outer tunnel (OT), inner sulcus, and TM. (E) In this *Dio2*^{-/-} cochlea, differentiation of the ISE and sensory epithelium has progressed to resemble the WT but the TM is still mildly enlarged. (F) This *Dio2*^{-/-} cochlea exhibits a grossly malformed TM and degeneration or aborted differentiation of the ISE and sensory epithelium (below asterisks). (Scale bars: 50 μ m for all.)

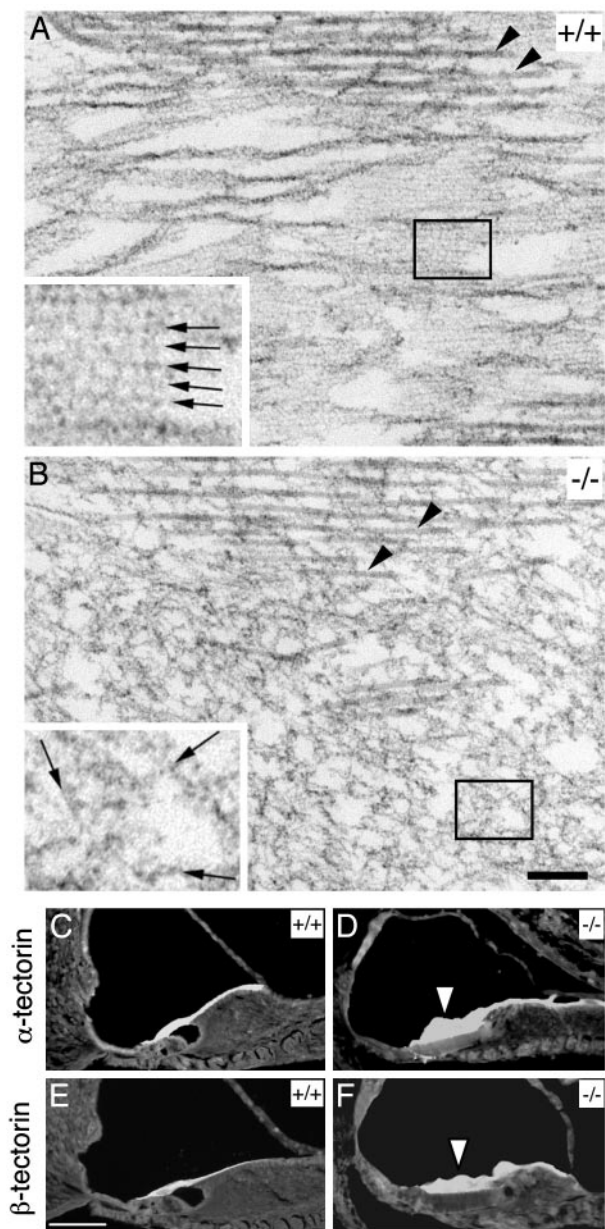


Fig. 4. TM defects in *Dio2*^{-/-} mice. (A) Transmission electron microscopy reveals the normal ultrastructure of a midregion of the TM, which contains major collagenous fibrils (arrowheads) and a fine, striated sheet matrix (parallel arrows in *Inset*). WT mouse, at P28. (B) In *Dio2*^{-/-} mice, the major collagen fibrils are present (arrowheads) but the fine filaments of the matrix are disorganized (arrows in *Inset*) in middle and upper regions of the TM (most distal to the inner sulcus). Small boxes indicate areas that are enlarged 3-fold in the *Inset*. (C–F) Immunofluorescent detection of tectorin components of the TM in WT (C and E) and *Dio2*^{-/-} (D and F) pups at P8. Note TM enlargement in *Dio2*^{-/-} mice (arrowhead). (Scale bars: 200 nm for A and B; 50 μ m for C–F.)

usually slight in adults although Fig. 3F shows an extreme example with a severely malformed TM and disorganization of the sensory epithelium, indicating degeneration or aborted development of these tissues.

The ultrastructure of the TM in *Dio2*^{-/-} mice was also abnormal. The normal TM contains a series of major collagen fibrils embedded in a striated sheet matrix of fine filaments (Fig. 4A) (34). In *Dio2*^{-/-} mice, the major fibrils were present but the striated sheet matrix was disorganized in the middle and upper

regions of the TM (Fig. 4B). The matrix, however, was normal in lower regions of the TM lying closest to the inner sulcus. The TM still contained the major α - and β -tectorin glycoproteins as found in WT mice (Fig. 4C) (30). This finding suggests, that as in TR-deficient mice, the lack of D2 does not block expression of TM components but instead may impair their assembly or processing. The cochlear defects in *Dio2*^{-/-} mice and the TM deformity with a partial disorganization of ultrastructure closely resembled the phenotype in TR β -deficient mice. The TM abnormalities, however, were milder than in mice lacking both TR β and TR α 1, in which ultrastructural disorganization occurred throughout all regions of the TM (13).

Recovery of Auditory Sensitivity by T3 Administration. The auditory phenotype in *Dio2*^{-/-} mice was remarkably similar to that caused by hypothyroidism despite the fact that *Dio2*^{-/-} mice possess serum levels of T4 and T3 that would normally be adequate for the development of hearing. This finding suggests that D2 is required to generate T3 in the auditory system above levels available in serum in postnatal development. Therefore, we tested whether T3 supplementation could overcome such a local T3 insufficiency in *Dio2*^{-/-} mice. T3 was added at different starting points to the drinking water of nursing *Dio2*^{+/-} dams or weaned progeny until P28 when the ABR was measured. Treatment from P10 with 0.3 μ g/ml T3 resulted in 2- to 5-fold increases in serum T3 when measured at P16 (Fig. 5B). After weaning at P21, mice that ingested T3 directly had 8- to 12-fold increased serum T3 levels ($n = 8$).

T3 given from P10 improved the mean ABR thresholds by ≈ 30 dB SPL in *Dio2*^{-/-} mice compared to untreated *Dio2*^{-/-} mice (Fig. 5A). The improvement was nearly complete in most *Dio2*^{-/-} mice although 30% showed only a limited recovery. A lesser degree of correction was achieved when T3 was given later. Treatment of *Dio2*^{-/-} pups from P16 improved ABR thresholds by ≈ 10 dB SPL, whereas treatment from P23 did not correct thresholds. T3 treatment of *Dio2*^{-/-} mice also enhanced the definition of the waveform (Fig. 5C and see untreated example in Fig. 2B) and improved its latency (with T3 treatment from P10, peak IV latency was shortened from 5.75 ± 0.19 to 4.87 ± 0.31 ms; $n = 8$; $P < 0.01$). Thresholds were in the normal range in *Dio2*^{+/-} or WT controls regardless of treatment.

Higher T3 doses produced systemic toxicity but paradoxically still improved ABR thresholds. T3 treatment (1 μ g/ml) begun *in utero* retarded body growth of *Dio2*^{-/-} pups, which attained $\approx 60\%$ of untreated weight at weaning (at P22, *Dio2*^{-/-} female treated, 5.8 ± 1.2 , untreated 9.6 ± 0.4 g; male treated, 4.9 ± 1.0 , untreated 9.2 ± 0.7 g; $n = 4-6$; $P < 0.001$). Mice in these treated groups showed ≥ 30 dB SPL improvements in ABR thresholds compared to untreated *Dio2*^{-/-} mice ($n = 14$; $P < 0.001$), although 30% (4 of 14) had only slight improvement. Treatment of *Dio2*^{+/-} control mice from embryonic stages, but not from P10 or later, also resulted in some progeny (3 of 12) acquiring a defective ABR. These findings suggest that the embryonic or neonatal auditory system is susceptible to impairment by excessive T3 regardless of genotype. The improvement of auditory sensitivity by T3 administration identified a T3 deficiency as the critical defect underlying the deafness in *Dio2*^{-/-} mice, in accord with the activity of D2 in generating T3.

T3 treatment also improved cochlear differentiation in *Dio2*^{-/-} mice. In untreated *Dio2*^{-/-} mice at P16 (Fig. 5D), differentiation had progressed from the immature state shown at P8 in Fig. 3B to form distinct hair cells and an open tunnel of Corti. However, the inner sulcus epithelium adjacent to the inner hair cells was thicker with a denser array of columnar cells than in *Dio2*^{+/-} pups and the TM remained malformed. In *Dio2*^{-/-} pups treated with T3 from P10 until analysis at P16, morphology more closely resembled the normal state for that stage of development with a better differentiated inner sulcus epithelium

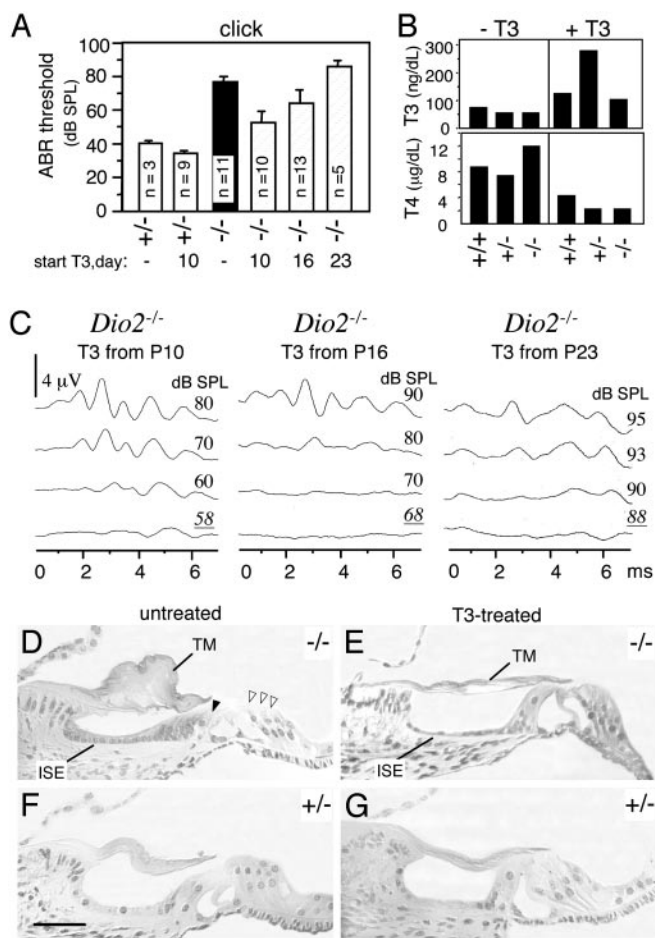


Fig. 5. T3 recovers auditory sensitivity in *Dio2*^{-/-} mice. (A) Mean ABR thresholds \pm SEM in mice at P28 after T3 treatment starting at P10, P16, or P23. Untreated groups are shown in columns marked -. T3 was given in drinking water (0.3 μ g/ml) to nursing dams and weaned progeny. Compared to untreated mice, thresholds were substantially corrected in *Dio2*^{-/-} progeny when treatment started at P10 ($P = 0.002$), partly corrected when started at P16 ($P = 0.005$), and not significantly corrected when started at P23 ($P = 0.06$). Similar results were obtained for 8-, 16-, and 32-kHz stimuli. (B) Serum levels of total T3 and total T4 in P16 pups after T3 treatment (0.3 μ g/ml) from P10. Treatment (+T3) increased T3 levels 2- to 5-fold over untreated levels (-T3). As expected, T3 treatment also suppressed the function of the pituitary-thyroid axis, thereby reducing T4 levels. Groups contained two pools of serum from 16–20 pups except for the T4 assays in +T3 groups, which contained single pools of serum from $n = 5$ –8 pups. (C) The thresholds and definition of ABR waveforms were improved in *Dio2*^{-/-} mice when treated from P10, but were less improved when treated from P16 and not improved when treated from P23. Thresholds are underlined. Traces are shown on a 4- μ V fixed scale. (D–G) Improved cochlear morphology in *Dio2*^{-/-} mice after T3 treatment from P10 until analysis at P16. In untreated *Dio2*^{-/-} pups (D), the TM is malformed and the inner sulcus epithelium (ISE) is thicker with taller, columnar cells than in untreated *Dio2*^{+/-} pups (F). T3 improved ISE differentiation and the shape of the TM in *Dio2*^{-/-} pups (E) but did not obviously alter morphology in *Dio2*^{+/-} control littermates (G). Inner and outer hair cells are marked by black and white filled arrowheads, respectively, in D. Sections show comparable apical turns of the cochlea. Groups, $n = 3$. (Scale bar: 50 μ m for D–F.)

and thinner TM (Fig. 5E). T3 treatment did not produce obvious abnormalities in *Dio2*^{+/-} control pups (Fig. 5F and G).

Discussion

This study reveals that D2 is essential for the development of hearing. The similarity of the cochlear phenotypes caused by D2 or TR deficiency suggests that D2 controls the T3 signal in the

cochlea to activate the TR at a critical phase of development. On a comparative scale, the auditory phenotype in *Dio2*^{-/-} mice is slightly worse than in TR β -deficient mice but is milder than that caused by deletion of both TR β and TR α 1 (13) or by severe systemic hypothyroidism (1). The differences in severity may be explained if the normal to slightly increased levels of circulating T3 in *Dio2*^{-/-} pups provide small amounts of ligand that stimulate limited activity by TR β and TR α 1 in the cochlea. In contrast, systemic hypothyroidism or the deletion of all TRs would cause a more absolute block of thyroid hormone signaling. The variable severity of some features of the *Dio2*^{-/-} phenotype is consistent with the enzymic role of D2 in generating subtle T3 fluxes in the cochlea, which may be expected to vary somewhat among individuals.

Why is the development of the auditory system so dependent on D2 and why would the maturing cochlea in effect stimulate its own response to thyroid hormone? The finding that normal or marginally increased serum levels of T3 are inadequate for the demands of the cochlea supports the role of D2 as a tissue-specific amplifier of T3 action. Thus, D2 would draw on the T4 supply in serum and convert this into T3 at the necessary stage of development. The demands for T3 in the postnatal cochlea may be considerable given the range of differentiation events that require thyroid hormone in the inner sulcus, sensory epithelium, spiral ganglion, and auditory nerve and in the formation of the TM (1, 3, 11, 13, 15). D2 may have other functions in central auditory pathways because D2 mRNA is also expressed widely in the brain, including in central auditory regions (24). Indeed, the poor overall definition and prolonged latency of the ABR waveform in *Dio2*^{-/-} mice raises the possibility that defects exist not only in the cochlea but also in the auditory nerve and brainstem auditory regions. Further study is necessary to determine whether there is any type of brain defect in *Dio2*^{-/-} mice. The improvement of auditory sensitivity by high T3 doses that otherwise retard body growth in *Dio2*^{-/-} pups emphasizes the differential demands of the auditory and other systems for T3 at certain developmental stages. Thus, the local amplification of the hormonal signal by D2 would provide an efficient means of coordinating multiple developmental events in the maturing auditory system. Moreover, the location of D2 in the auditory system itself may confer immediacy in prompting the timely maturation of this sensory system.

The results suggest that T3 signaling regulates not only the onset of auditory function but also the subsequent maturation of auditory sensitivity. The maturation of auditory function in rodents occurs over a protracted period lasting until \approx P20 (6, 7). A continuing role for D2 over this period would explain why T3 administration begun as late as P16 partly improves auditory sensitivity in *Dio2*^{-/-} mice. Possibly, some functions are simply arrested and the later provision of T3 is able to reactivate these differentiation events. The chambered structure of the cochlea points to the likelihood that D2 function is integrated with currently unknown mechanisms that would transport specific forms of thyroid hormones across the compartments of the cochlear microenvironment. A paracrine-like action of thyroid hormone may therefore operate involving T4 uptake, the generation of T3 by D2, and the release of this T3 to stimulate responsive tissues in the cochlear interior.

D3, a thyroid hormone-inactivating enzyme, has been shown to regulate *Xenopus* eye development, probably by reducing local levels of thyroid hormone in the dorsal retina (25). Our results suggest that the hormone-activating D2 enzyme provides an equally potent developmental control but through an opposite mechanism of amplifying T3 action. Thus, both enzymes may promote critical tissue-specific variations in local T3 levels in development. Somewhat analogously, the enzymes that synthesize and degrade retinoic acid are thought to control the activity of retinoid receptors (35). The findings in *Dio2*^{-/-} mice suggest

that the amplification of thyroid hormone action in the target organ itself provides an important developmental control in the auditory system.

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