Deletion of the Thyroid Hormone–Activating Type 2 Deiodinase Rescues Cone Photoreceptor Degeneration but Not Deafness in Mice Lacking Type 3 Deiodinase

Lily Ng,1 Hong Liu,1 Donald L. St. Germain,2 Arturo Hernandez,2 and Douglas Forrest1

1Laboratory of Endocrinology and Receptor Biology, National Institute of Diabetes and Digestive and Kidney Disorders, National Institutes of Health, Bethesda, Maryland 20892; and 2Maine Medical Center Research Institute, Scarborough, Maine 04074

Type 2 deiodinase amplifies and type 3 deiodinase depletes levels of the active form of thyroid hormone, triiodothyronine. Given the opposing activities of these enzymes, we tested the hypothesis that they counteract each other’s developmental functions by investigating whether deletion of type 2 deiodinase (encoded by Dio2) modifies sensory phenotypes in type 3 deiodinase-deficient (Dio3−/−) mice. Dio3−/− mice display degeneration of retinal cones, the photoreceptors that mediate daylight and color vision. In Dio2−/− mice, cone function was largely normal but deletion of Dio2 in Dio3−/− mice markedly recovered cone numbers and electroretinogram responses, suggesting counterbalancing roles for both enzymes in cone survival. Both Dio3−/− and Dio2−/− strains exhibit deafness with cochlear abnormalities. In Dio3−/−; Dio2−/− mice, deafness was exacerbated rather than alleviated, suggesting unevenly balanced actions by these enzymes during auditory development. Dio3−/− mice also exhibit an atrophic thyroid gland, low thyroxine, and high triiodothyronine levels, but this phenotype was ameliorated in Dio3−/−; Dio2−/− mice, indicating counterbalancing roles for the enzymes in determining the thyroid hormone status. The results suggest that the composite action of these two enzymes is a critical determinant in visual and auditory development and in setting the systemic thyroid hormone status. (Endocrinology 158: 1999–2010, 2017)

Mammalian development is promoted by a progressive rise in circulating levels of triiodothyronine (T3) and thyroxine (T4), a tetra-iodinated precursor of T3. However, tissues respond to T3 in distinct ways and with different time courses. This individualized response may be determined partly in the tissue itself by the amplification or depletion of T3 levels by type 2 and type 3 deiodinases, respectively (1–4). Type 2 deiodinase (encoded by Dio2) converts T4 into T3 by outer ring 5β-deiodination whereas type 3 deiodinase (encoded by Dio3) depletes T4 and T3 by inner ring 5-deiodination, producing reverse T3 and 3,3′-diiodothyronine, respectively.

The functions of the Dio3 gene include a prominent role in sensory development. Dio3−/− mice lose retinal cones, the photoreceptors that mediate daylight vision and color vision (5) and represent a model for achromatopsia, a form of retinal degeneration with loss of cone function (6). Dio3−/− mice also exhibit deafness and premature cochlear differentiation, suggesting a protective role for type 3 deiodinase during auditory development (7). Additionally, Dio3−/− mice display retarded growth and an atrophic thyroid gland with abnormal T4 and T3 levels, reflecting defects in the pituitary–thyroid axis and in the systemic thyroid hormone status (8, 9). Dio2−/− mice exhibit a different array of phenotypes, including moderately elevated T4 levels and impaired negative feedback by T4 over the pituitary–thyroid axis (10, 11). Dio2−/− mice also...
display deafness with cochlear retardation similar to that caused by hypothyroidism, despite having circulating T4 and T3 levels that would normally suffice for the development of hearing (12).

The opposing activities of type 2 and type 3 deiodinases suggest that their net action may be a key determinant of development. This composite action is likely to be dynamic given the shifting, or reciprocal, patterns of expression of these deiodinases in many immature tissues (1, 13, 14), including the cochlea (7, 15). The net action may also involve a role in setting levels of T4 and T3 in the circulation, which may further modify tissue outcomes. To test whether the Dio2 and Dio3 genes counteract each other in development, we investigated whether deletion of Dio2 reverses or otherwise modifies visual and auditory phenotypes in Dio3−/− mice.

Materials and Methods

Mouse strains

Strains carrying Dio2 (10) and Dio3 (8) knockout alleles were crossed to generate wild-type (WT), Dio2−/−, Dio3−/−, and Dio2−/−; Dio3−/− groups on a similar genetic background (C57BL/6) × 129/Sv. Dio3−/−;Dio2−/− parents were crossed to generate groups for analyses. Genotyping was determined by polymerase chain reaction (PCR) as described (8, 12). Studies on mice followed American Thyroid Association guidelines (16) and approved protocols at the National Institute of Diabetes and Digestive and Kidney Diseases at the National Institutes of Health. Most results are presented for mixed groups of males and females, unless otherwise noted. The low survival rates of mixed groups of males and females, unless otherwise noted. The survival rates of Dio3−/− pups (8) and the complex breeding scheme necessary to generate requisite genotypes made it impractical in most cases to obtain sufficient numbers for groups of only one gender. There were no obvious gender-based differences in the phenotypes examined. For serum hormones in adults, separate male and female groups are presented.

Immunohistochemistry and in situ hybridization

Eyes were dissected and fixed in 4% paraformaldehyde/phosphate-buffered saline (PBS) for 3 to 5 hours at 4°C, then rinsed in PBS, cryoprotected in 30% sucrose, and embedded in OCT. For immunostaining for opsins or activated caspase 3, 10-μm-thick cryosections were incubated overnight with primary antibody in PBS with 1.5% normal goat or horse serum, 0.1% bovine serum albumin, and 0.4% Triton X-100, as described (5). Sections were rinsed in PBS then incubated with second antibody for color development (Vector Laboratories, Burlingame, CA) or with Alexa Fluor 488 goat anti-rabbit or anti-mouse secondary antibody for immunofluorescence. For in situ hybridization, eyes were fixed in 4% paraformaldehyde/PBS overnight. Cryosections were incubated with digoxigenin-labeled riboprobes made from a mouse Dio2 complementary DNA (cDNA), spanning bases 590 to 1383 downstream of the ATG start codon, as described (12). For antibodies used, see Table 1.

RNA analysis

RNA sequencing datasets represent pools of six to 12 retinas from WT mice (of mixed C57BL/6 × 129/Sv background) at the stages shown in Fig. 1. An independent analysis on a separate series of samples [at embryonic day 17, postnatal day (P)6, and P14] gave similar results. Total RNA was prepared using TRIzol reagent and messenger RNA (mRNA) enriched using a Dynabeads purification kit (Thermo Fisher, Grand Island, NY). cDNA was synthesized using a SuperScript double-strand cDNA kit (Thermo Fisher) then fragmented with a Bioruptor (Diagenode, Danville, NJ) with medium output settings for 15 minutes. Libraries were constructed using a TruSeq chomatin immunoprecipitation library preparation kit (Illumina, San Diego, CA). Fifty base single-end reads were generated on an Illumina HiSeq 2500 sequencer, and prealignment of data was performed at the National Institute of Diabetes and Digestive and Kidney Diseases Genomics Facility and further analyzed using a Genomatix (Munich, Germany) platform. Data were converted using bcl2fastq software version 2.17.1.14 (Illumina). After chastity filtering, alignment was performed using BBMap version 36.02 to mouse MM9 reference genome. The accession number for the WT retina datasets on the Gene Expression Omnibus database is GSE95016.

For reverse transcription polymerase chain reaction (RT-PCR), cDNA was made from retinal RNA from WT and Dio2−/− male mice at P28. Analysis of Dio2 and Gapdh genes was performed with primers as follows: Dio2 forward, 5′-CAG GTA ACA ATT ATG CCT CCG-3′, reverse, 5′-GCT GAA ATT CTT CTC CAG CCA AAC-3′; Gapdh forward, 5′-TGG TGA ACG GAT TTT GCC GTA-3′, reverse, 5′-AGT GAC TGT GGT CAT GAG CCC TTC-3′. Both product bands were 500 bp.

Histology

Eyes were fixed in 2% paraformaldehyde/3% glutaraldehyde in PBS for 24 hours. The lens was removed and then the retina was rinsed in PBS and dehydrated in an ethanol gradient

| Antibody | Peptide/Protein Target | Species | Type | Manufacturer | Catalog No. | Dilution | RRID |
|----------|------------------------|---------|------|--------------|-------------|----------|-------|
| Anti-5 opsin | 5 opsin | Goat | Polyclonal | Santa Cruz Biotechnology | Sc-14363 | 1:1000 | AB_2158332 |
| Anti-M opsin | M opsin | Rabbit | Polyclonal | Millipore | AB 5405 | 1:1000 | AB_177456 |
| Anti-activated caspase 3 | Caspase 3 | Rabbit | Polyclonal | Promega | G748A | 1:300 | AB_430875 |
| Anti-glutamine synthetase | Glutamine synthetase | Mouse | Monoclonal | Millipore | MAB302 | 1:300 | AB_2110656 |

Abbreviation: RRID, Research Resource Identifier.
before embedding in glycol methacrylate plastic (5); 3-μm-thick microtome sections were stained with Gill no. 3 hematoxylin with eosin. Cochlea were fixed for 1 to 2 days, then immersed in 0.1 M EDTA for 1 week before embedding in plastic; 6-μm-thick sections were stained with Gill no. 3 hematoxylin. Areas of the gland and follicles were measured using ImageJ and are expressed as arbitrary units (1 unit = 12.3 mm²) for five sections per gland for three to four mice. Cone and rod nuclei were counted in 208-μm-long fields of the outer nuclear layer, on three to four sections per mouse for three mice.

**Statistical analysis**

Statistical significance between several genotype groups was determined by one-way analysis of variance (ANOVA) and a Tukey post hoc test using GraphPad Prism software. A Student t test was used for other two group comparisons.

**Deiodination assays and hormone measurements**

Assays for type 2 deiodinase activity and for T3 and T4 levels were performed as described (8, 18). Pools of dissected eyes, retinae, or cochleae were homogenized in buffer containing 20 mM Tris-HCl (pH 7.6), 5 mM dithiothreitol, and 0.25 mM sucrose. Pools of four to six eyes, six to 10 retinae, and eight cochleae were analyzed in triplicate. Homogenates were incubated with [125I]T4 substrate, 1.2 mM EDTA, 1 mM propylthiouracil, 1 mM T3, and 20 mM dithiothreitol for 1 hour at 37°C, and then products were separated using Bio-Rad AG 50WXG (H+) resin to measure [125I]I product. Serum total T4 and T3 values were determined by radioimmunoassay using Coat-A-Count reagents (Diagnostic Systems Laboratories, Webster, TX), as described (8). Hormones were measured for individual adult mice and for pools of two to three pups due to limited volumes of serum obtained.

**Results**

**Dio2 expression in mouse retina**

Given the role of type 3 deiodinase in cone photoreceptor function (5), we further investigated type 2 deiodinase in the retina in mice. RNA sequencing analysis revealed rising levels of Dio2 mRNA in the postnatal retina [Fig. 1(a)]. Dio3 mRNA displayed an...
earlier peak, as expected (5), suggesting a reciprocal developmental profile for Dio3 and Dio2 expression. Little or no Dio1 mRNA (type 1 deiodinase) was detected at any stage.

Type 2 5'-deiodination activity was detected in homogenates of WT mouse eye or dissected retina at postnatal ages [Fig. 1(b)]. This activity was attributed to type 2 deiodinase because the activity was abrogated in Dio2−/− mice and was resistant to inhibition by propylthiouracil, thereby distinguishing it from type 1 deiodinase activity. The type 2 deiodination activity in the eye or retina was low compared with that in the cochlea at P8, a known enriched source of type 2 deiodinase in mice (15). RT-PCR analysis also detected Dio2 mRNA in retina in WT mice but not in Dio2−/− mice [Fig. 1(c)].

In situ hybridization detected Dio2 mRNA in the postnatal retina in the inner nuclear layer [Fig. 1(d)], which contains several types of interneurons and Müller glia. A specific Dio2 signal was not detected on sections from Dio2−/− mice, which lack the portion of the gene used as a probe for in situ hybridization (12). The Dio2 signal was weak but localized to an irregular band of cells in a central zone of the inner nuclear layer, suggestive of expression in Müller glia. This proposal was supported by double staining for glutamine synthetase, a marker of Müller glia [Fig. 1(e)].

**Rescue of cone photoreceptors in Dio3−/−;Dio2−/− mice**

To test whether deletion of Dio2 resulted in cone defects or modified the phenotype in Dio3−/− mice, we examined opsin expression. Most mammals express two cone opsin photopigments with peak sensitivity to medium-long (M, or green) and short (S, or blue) wavelengths of light (19). In WT mice, opsins are distributed in gradients: M opsin predominates in cones in the superior retina, and S opsin predominates in the inferior retina [Fig. 2(a) and 2(b)]. Dio3−/− adult mice lacked most cones of both M and S types (5), but Dio2−/− mice displayed no obvious opsin abnormality. However, Dio3−/−;Dio2−/− mice recovered M and S opsin expression compared with Dio3−/− mice [Fig. 2(c)]. The opsin gradient was slightly exaggerated in Dio3−/−;Dio2−/− mice: M opsin was poorly restored in inferior regions, and S opsin was poorly restored in superior regions, suggesting that although cones were rescued, opsin patterning was not fully normal in the absence of both deiodinases.

Histological analysis corroborated the recovery of cones in Dio3−/−;Dio2−/− mice. In mice, ~3% of photoreceptors

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*Figure 2.* Immunohistochemistry for cone opsins in deiodinase-deficient mice. (a) Diagram of a vertical section through the eye depicting the superior and inferior views of the retina shown in (b). The retina is gray-shaded at the back of the eye. (b) In WT mice at 2 months of age, M opsin predominates in cones in superior (sup) and S opsin in inferior (inf) regions of the retina (arrowheads indicate stained cone segments). Dio3−/− mice lack both cone types, but Dio3−/−;Dio2−/− mice regain many M and S cones. IS/OS, inner and outer segments; ONL, outer nuclear layer. (c) Counts of M and S opsin–positive cones. Groups contained three mice. ANOVA was performed for all genotypes. **P < 0.01, ***P < 0.001, for comparisons of mutant genotypes to WT; #P < 0.05, ###P < 0.001, for comparisons of Dio3−/−;Dio2−/− to Dio3−/−.
are cones and ~97% are rods, the photoreceptor type that mediates vision in dim light (20). Cones are distinguished by their location at the edge of the outer nuclear layer and by their large nuclei compared with rods, which have small, dense nuclei [Fig. 3(a)]. In Dio3−/−:Dio2−/− mice, cone nuclei attained ~70% of WT numbers [Fig. 3(b)]. Dio2−/− mice displayed normal numbers of cone and rod nuclei. Dio3−/− and Dio3−/−:Dio2−/− genotypes both displayed a modest loss of rods (~12% below WT), suggesting a minor vulnerability of rods in these genotypes.

Cell death in the postnatal retina

In Dio3−/− mice, cones degenerate by apoptosis in the postnatal period (5), as demonstrated by staining for activated caspase 3 [Fig. 4(a)]. In WT pups, a modest incidence of apoptosis occurs with a peak at ~P2 and then a decline to low levels during the next 2 weeks (21). Apoptotic cells appear in shifting patterns in neonatal mice, as many immature cell types have not yet reached their final location. Cones migrate inward through the neuroblast layer and then outward to the outer zone of the outer nuclear layer as the retina matures (22). At P2, Dio3−/−:Dio2−/− mice displayed fewer caspase 3–positive cells in the neuroblast layers than do Dio3−/− mice (Fig. 4). In Dio2−/− mice, caspase 3–positive cell numbers were in the normal range.

ERG analysis

Dio3−/− mice display a defective cone ERG in response to photopic stimuli at 520 nm and 360 nm (5), wavelengths that optimally activate M and S opsins, respectively, in mice (23) [Fig. 5(a)]. Dio2−/− mice had normal responses at both wavelengths. However, compared with Dio3−/− mice, Dio3−/−:Dio2−/− mice recovered substantial responses at both wavelengths, and the ERG b-wave magnitudes approached normal over a range of stimulus intensities [Fig. 5(b)].

We observed a reduced rod ERG in Dio3−/− mice as expected (5) and in Dio2−/− mice, suggesting that rod function was modestly impaired in the absence of either type 3 or type 2 deiodinase. In Dio3−/−:Dio2−/− mice, the rod b-wave magnitude was somewhat increased compared with Dio3−/− mice and approached the level in Dio2−/− mice.

ABRs

Both Dio3−/− and Dio2−/− mouse strains exhibit deafness with a defective ABR (7, 12). Given that both deiodinases are expressed in the immature cochlea, we tested whether deletion of Dio2 would ameliorate auditory defects in Dio3−/− mice. In Dio3−/− and Dio2−/− adult mice, auditory thresholds were elevated for a click (broadband frequency) stimulus [Fig. 6(a)]. Thresholds were 68 ± 5 and 76 ± 3 dB sound pressure level (SPL) for Dio3−/− and Dio2−/− genotypes, respectively, and 46 ± 2 dB SPL for WT [mean ± standard error of the mean (SEM)] [Fig. 6(a)]. However, in Dio3−/−:Dio2−/− mice, the threshold was 97 ± 2 dB SPL, worse than for either single mutant (P < 0.001). Dio3−/−:Dio2−/− mice displayed similarly exacerbated defects in response to pure tone stimuli at 8, 16, and 32 kHz frequencies that span the sensitive range of hearing in mice (not shown).

Figure 6(b) shows representative waveform traces with three to four peaks detected within 5 ms of the stimulus in WT mice. In contrast, in many Dio3−/−:Dio2−/− mice, no specific peaks could be evoked even with a maximal stimulus at 100 dB SPL. The waveforms that were evoked in the single mutant strains displayed significantly lower
magnitudes than in WT mice when subjected to equivalent stimuli [Fig. 6(c)].

**Cochlear morphology**

The cochlear abnormalities in *Dio3*<sup>−/−</sup> (7) and *Dio2*<sup>−/−</sup> (12) mice suggest that type 3 deiodinase constrains and type 2 deiodinase stimulates the rate of postnatal cochlear remodeling prior to the onset of hearing (at P13). In WT mice at P9, the organ of Corti, which contains the mechanosensory hair cells, approaches a nearly mature morphology with three rows of outer hair cells and one row of inner hair cells on either side of an open tunnel of Corti [Fig. 7(a)]. The hair cells are elongated above the Deiters support cells, and the greater epithelial ridge, a transient cell-dense structure, has largely regressed to form the inner sulcus cavity below the tectorial membrane. The tectorial membrane extends to the hair cells and is essential for auditory transduction (24). The morphological status of the organ of Corti in *Dio3*<sup>−/−</sup> pups is accelerated in the first few neonatal days, but by P9, it appears relatively normal whereas in *Dio2*<sup>−/−</sup> pups, the morphology is retarded. In *Dio2*<sup>−/−</sup> pups, the greater epithelial ridge displayed only initial signs of regression, the hair cells lacked an upright structure, the tunnel of Corti was unopened, and the tectorial membrane was enlarged. *Dio3*<sup>−/−</sup>*;Dio2*<sup>−/−</sup> mice displayed a similar retardation as in *Dio2*<sup>−/−</sup> mice. Thus, deletion of *Dio2* resulted in retardation or over-reversal rather than correction of the *Dio3*<sup>−/−</sup> phenotype.

In adults, the morphology in *Dio3*<sup>−/−</sup>*;Dio2*<sup>−/−</sup> mice still resembled that in *Dio2*<sup>−/−</sup> mice: in both genotypes, overall morphology had caught up with that in WT mice but the tectorial membrane was persistently enlarged.

**Thyroid hormones and thyroid gland morphology**

In addition to defects in the retina and cochlea, *Dio2*<sup>−/−</sup> (10) and *Dio3*<sup>−/−</sup> (8) mice have abnormalities in systemic T4 and T3 levels that could contribute to phenotypes. Therefore, we investigated whether deletion of both genes in *Dio3*<sup>−/−</sup>*;Dio2*<sup>−/−</sup> mice modified serum levels of T4 and T3. *Dio3*<sup>−/−</sup> pups are known to exhibit an extreme phenotype of undetectable T4 and high T3 (8), but in *Dio3*<sup>−/−</sup>*;Dio2*<sup>−/−</sup> pups at P5, T4 was increased and T3 decreased to approximately normal ranges [Fig. 8(a)]. *Dio2*<sup>−/−</sup> pups had a high T4 level, as expected from previous evidence at juvenile ages (10, 12). By weaning and adult ages, *Dio3*<sup>−/−</sup> mice eventually acquire detectable levels of T4, although still below normal, whereas T3 drops to subnormal levels (8). *Dio2*<sup>−/−</sup> mice retain slightly high T4 beyond weaning age (10, 12). In *Dio3*<sup>−/−</sup>*;Dio2*<sup>−/−</sup> mice at 2 to 3 months of age, both T4 and T3 were in the normal range [Fig. 8(b)], suggesting a persistent stabilization of T4 and T3 levels in the circulation.
In **Dio3**^{−/−};**Dio2**^{−/−} mice, the thyroid gland recovered a more normal size and follicular content than did the atrophic gland in **Dio3**^{−/−} mice (8) [Fig. 8(c) and 8(d)]. In **Dio2**^{−/−} mice, the gland was slightly enlarged. In WT or **Dio3**^{−/−};**Dio2**^{−/−} mice, most follicles (69% and 62%, respectively) were medium-sized (100 to 200 area units) or larger. In contrast, in **Dio3**^{−/−} mice, most follicles (70%) were small (<100 area units), and only 30% were medium-sized or larger. In **Dio2**^{−/−} mice, the distribution of follicle sizes resembled that in WT mice. The results indicate counteracting roles for **Dio3** and **Dio2** genes in determining thyroid gland morphology and systemic levels of T4 and T3.

**Developmental weight gain**

**Dio3**^{−/−};**Dio2**^{−/−} mice thrived reasonably well and gained more weight than **Dio3**^{−/−} mice at juvenile ages (8). At 5 weeks of age, weights of WT and **Dio3**^{−/−} males were 18.5 ± 1.7 and 11.5 ± 3.7 g, respectively (mean ± SEM; n = 7; P < 0.001). **Dio3**^{−/−};**Dio2**^{−/−} males weighed 15.3 ± 2.6 g, representing a 30% improvement compared with **Dio3**^{−/−} males (n = 10, P = 0.01). Recovery of weight continued into adulthood. Young females displayed similar trends, but after 5 weeks of age, both **Dio3**^{−/−};**Dio2**^{−/−} and **Dio3**^{−/−} genotypes were of similar weight, and neither was significantly different than WT female mice (n = 6, P = 0.9).

**Discussion**

This study shows that deletion of **Dio2** strongly modifies phenotypes in **Dio3**^{−/−} mice and supports the proposal that the composite action of both type 2 and type 3 deiodinases is critical for mammalian development. We suggest that such combined action offers enhanced control over T3 functions in sensitive tissues during development. The outcomes vary between tissues and may involve both direct and indirect influences to different extents at distinct stages, as discussed later.

**Retinal photoreceptors**

T3 controls the differentiation and survival of cone photoreceptors. The expression of M opsin and the patterning of M and S opsins over the retina depend on a
cone-specific thyroid hormone receptor (25) and adequate T3 and T4 in the circulation (26–29). However, an excess of T3 or deletion of Dio2 leads to cone cell death, indicating that the T3 signal must also be appropriately constrained (5). Our findings on the Dio2 gene suggest a possible additional need for amplification of T3 in the postnatal retina.

A role for type 2 deiodinase in the mammalian retina was implied by an earlier report of outer ring deiodinase activity in the rat retina (30). In the postnatal mouse retina, we detected type 2 deiodination, although at low levels. In situ hybridization localized Dio2 mRNA in Müller glial cells, in agreement with a report that mentioned detection of Dio2 mRNA by microarray analysis in flow-sorted Müller cells (31). Moreover, we detected little or no Dio2 mRNA in isolated cones and rods (unpublished data), cell types that constitute ~70% of the retinal cell population. These findings suggest similarities between the retina and the brain, in which Dio2 is expressed mostly in glia rather than neurons (32). Müller glial projections span the layers of the retina from the outer limiting membrane to the inner surface of the ganglion cell layer and form an interface with the vasculature that provides homeostatic support for photoreceptors and other retinal neurons (33). Thus, in addition to their role in intercellular transfer of ions and metabolites, Müller glia may form part of a cellular pathway for transfer of T3 in the retina.

Dio2−/− mice had no overt cone defects but did have a moderately impaired rod ERG, suggesting a subtle role for type 2 deiodinase on rod function. We previously noted a reduced rod ERG in Dio3−/− mice (5). We cannot exclude a subtle role for type 2 deiodinase on nonphotoreceptor cell types or on nondevelopmental processes such as aging. However, the major impact of deletion of Dio2 was revealed in Dio3−/− mice, which resulted in rescue of cones. This rescue potentially reflects adjustments both of locally generated T3 in the retina and of the high systemic T3 levels that occur in young Dio3−/− mice. Future investigation of these possibilities may be aided by cell-specific deletions of Dio2 and Dio3 genes.

A Dio2 knockout in zebrafish (34) reduced the size of the eye and ear, suggesting a conserved role for the Dio2 gene in these sensory organs. Knockdown of Dio2 or Dio3 in zebrafish also delayed growth of the eye, delayed opsin expression, and partially disorganized the retinal layers (35). Both Dio2 and Dio3 genes are also expressed in the chick embryonic retina (36), suggesting that a composite action by both enzymes in the retina may be widely conserved.

The recovery of cones in Dio3−/− mice following the deletion of Dio2 may have wider implications for retinal degeneration and loss of vision (37). Most known forms of retinal degeneration do not result from defective T3 signaling, although a high free T4 level in adult human populations has been associated with an increased risk of age-related macular degeneration (38), and cone defects arise in rare cases of resistance to thyroid hormone (39). However, in mouse models of achromatopsia (Pde6c mutant) and Leber congenital amaurosis (Rpe65 mutant) that do not originate from disrupted T3 signaling, the loss of cones at juvenile ages was reported to be partly reduced by chemically induced hypothyroidism (40) or virus-mediated overexpression of Dio3 (41). Thus, T3 may be a secondary influence over photoreceptor degeneration caused by other factors. Deiodinases may
offer a potential target for the design of interventions to ameliorate the loss of photoreceptors.

**Auditory system**

The development of hearing requires T3 (42), and deafness is a risk in endemic iodine deficiency, congenital hypothyroidism (43), and resistance to thyroid hormone (44). The expression patterns of Dio3 and Dio2 genes in the mouse cochlea indicate that substantial control over auditory development is governed by deiodination within the auditory system (7). Our study demonstrates that deletion of Dio2 in Dio32/2 mice worsens rather than neutralizes the deafness in Dio32/2 mice. Thus, in the auditory system, deiodinases do not evenly balance each other’s function and may serve partly independent roles. In Dio32/2; Dio22/2 mice, cochlear morphology is retarded as in Dio22/2 mice, which may be interpreted as an over-reversal of the premature differentiation in Dio32/2 mice.

It is noteworthy that the deafness in Dio32/2; Dio22/2 mice is exacerbated despite circulating levels of T4 and T3 that should suffice for auditory development, implying that altered deiodination within the auditory system is a major cause of the phenotype. We speculate that the severe phenotype in Dio32/2; Dio22/2 mice may involve a double hit at different times or in different cell types in the auditory system. Not all defects need reside in the cochlea. T3 controls cochlear remodeling and function (42, 43) but also promotes the maturation of auditory relay centers in the brainstem (46), in which Dio2 mRNA is detected in rat pups (47). T3 also promotes myelination of the eighth nerve (48) and maturation of the ossicles and middle ear (49). In certain brain regions, type 2 deiodination is thought to generate T3 (50), whereas type 3 deiodination suppresses T3 responses (51). Conceivably, both processes promote the maturation of the central auditory pathway.

The cellular pathway that transfers T3 within the compartmentalized cochlea is poorly understood but presumably involves the coordinated action of deiodinases and membrane transporters for the uptake and release of T4 or T3 by specific cell types (52). However, the definition of these cell types and their anatomical proximity to cells that respond to T3 awaits improved methods for detection of deiodinases with precise cellular resolution.

**Thyroid hormone status and other implications**

In addition to tissue-intrinsic consequences of deletion of type 2 deiodinase in modifying phenotypes in Dio32/2 mice, there may be an indirect influence of the relatively normalized systemic levels of T4 and T3. This could result from known functions of the Dio2 gene in the hypothalamic–pituitary–thyroid axis, perhaps consistent with the rescued thyroid gland morphology in Dio32/2; Dio22/2 mice. Deletions in mice indicate a role for type 2 deiodinase in the anterior pituitary in the control of thyrotropin production and in the mediobasal hypothalamus in the control of thyrotropin-releasing hormone (10, 53). In the neonatal rat hypothalamus, a rise of

**Figure 7.** Cochlear histology in deiodinase-deficient mice. (a) At P9, a few days before hearing begins in WT mice, the organ of Corti is not fully mature but displays an open tunnel of Corti (tc) between the inner pillar (ip) and outer pillar (op) cells. The inner and outer hair cells (gray-filled and open arrowheads, respectively) are elongated, and the inner sulcus (is) is open beneath the tectorial membrane (tm). Morphology is similar in WT and Dio32/2 mice, but in Dio22/2 and Dio32/2; Dio22/2 genotypes it is retarded: the tunnel of Corti has not opened (black arrow), the hair cells have not elongated, the inner sulcus is only partly open (*), many cells remain in the greater epithelial ridge (ger), and the tectorial membrane is enlarged (gray arrow). (b) In adults (2 to 3 months old), the morphology in Dio22/2 and Dio32/2; Dio22/2 genotypes has largely caught up with that in WT and Dio32/2 mice, but the tectorial membrane remains enlarged.

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activating deiodination follows an earlier peak of inactivating deiodination, suggesting that functional maturation of the hypothalamic–pituitary–thyroid axis may require the balanced activities of both type 3 and type 2 deiodinases (1). Immunohistochemical studies also suggest that both deiodinases are expressed in regions of the human hypothalamus (54, 55). However, other peripheral means may modify the systemic thyroid hormone status. Combined deletions of Dio2 and Dio1 (56) or of all three Dio3, Dio2, and Dio1 deiodinase genes (57) suggest that peripheral clearance by type 1 or type 3 deiodinase modifies the levels of T4 and T3 in the circulation.

In summary, our study suggests that the composite action of the Dio3 and Dio2 genes is critical for visual and auditory development and for setting the thyroid hormone status. Dio3−/− male mice also display hypogonadism that can be partly rescued by deletion of the Dio2 gene (58). Dual patterns of Dio3 and Dio2 expression characterize the immature retina, cochlea (7), brain regions (1), skeletal muscle (4), and brown adipose tissue (59) in mammals and also occur in nonmammalian species (60, 61), suggesting that together these genes provide a widely adaptable developmental mechanism.

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Address all correspondence and requests for reprints to: Douglas Forrest, PhD, National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Diseases, 10 Center Drive, Building 10, MSC-1772, Bethesda, Maryland 20892. E-mail: forrestd@niddk.nih.gov.

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