Published in final edited form as:

Mol Cell Endocrinol. 2015 May 15; 407: 1–8. doi:10.1016/j.mce.2015.02.025.

The timecourse of apoptotic cell death during postnatal remodeling of the mouse cochlea and its premature onset by triiodothyronine (T3)

R. P. Peeters^{1,2,3,*}, L. Ng^{3,*}, M. Ma³, and D. Forrest³

¹Dept. of Internal Medicine, Erasmus Medical Center, Rotterdam, Netherlands ²Rotterdam Thyroid Center, Erasmus Medical Center, Rotterdam, Netherlands ³Laboratory of Endocrinology and Receptor Biology, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892, USA

Abstract

Apoptosis underlies various forms of tissue remodeling during development. Prior to the onset of hearing, thyroid hormone (T3) promotes cochlear remodeling, which involves regression of the greater epithelial ridge (GER), a transient structure of columnar cells adjacent to the mechanosensory hair cells. We investigated the timecourse of apoptosis in the GER and the influence of ectopic T3 on apoptosis. In saline-treated mice, activated caspase 3-positive cells were detected in the GER between postnatal days 7 and 13 and appeared progressively along the cochlear duct from base to apex over developmental time. T3 given on P0 and P1 advanced the overall program of apoptosis and remodeling by ~4 days. Thyroid hormone receptor β was required for these actions, suggesting a receptor-mediated process of initiation of apoptosis. Finally, T3 given only at P0 or P1 resulted in deafness in adult mice, thus revealing a transient period of susceptibility to long-term damage in the neonatal auditory system.

Keywords

thyroid hormone receptor; thyroid hormone; auditory system; apoptosis; organ of Corti

1.1 Introduction

Auditory function in mammals requires the formation of an array of mechanosensory hair cells and supporting cells in the organ of Corti of the cochlea. In mice, the different cell types and overall structure of the cochlea are formed by the time of birth (1, 2) but the cochlea continues to mature structurally and functionally before hearing begins at ~2 weeks

© 2015 Published by Elsevier Ireland Ltd.

*These authors contributed equally as first authors.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

of age (3, 4). A prominent structural remodeling event is the regression of the greater epithelial ridge (GER, also known as Kölliker's organ), a mass of tall columnar cells that resides next to the sensory hair cell array in the immature cochlea. Remodeling of the GER creates the inner sulcus, a cavity that allows the hair cell stereocilia to move dynamically against the tectorial membrane during auditory transduction (5). Prior to regression, GER cells secrete glycoprotein components of the tectorial membrane (6) and may provide cellular signals that promote the differentiation of the adjacent sensory epithelium (2).

The signals that drive remodeling of the GER and its developmental timing are poorly understood. In a histological study in the cat, Hinojosa (7) described a "transformation" that replaced the densely-packed columnar cells of the GER with cuboidal cells that form the inner sulcus epithelium. Subsequent observations at selected ages noted that the GER contained cells positive for the apoptosis marker TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labeling) at postnatal day 6 (P6) in Wistar rats (8) and at P7 and P10 in C3H/HeJ mice (9). These findings suggested an involvement of programmed cell death in the regression of the GER but the developmental onset and progression of this apoptosis are incompletely defined.

Thyroid hormone is critical for the development of hearing (10). In rodents, developmental hypothyroidism (11–17) or mutation of the *Thrb* thyroid hormone receptor β gene (18–20) retard the remodeling of the GER and cause deafness. In humans, endemic iodine deficiency (21), early developmental hypothyroidism (22) and *THRB* mutations (23, 24) are associated with hearing loss. Here, we report the timecourse of apoptosis in the cochlea in mice over the first two postnatal weeks by analysis of activated caspase 3, a cysteine protease effector of apoptosis, and TUNEL. Furthermore, since thyroid hormone is known to promote apoptosis during remodeling in tissues such as the brain and tail in amphibian metamorphosis (25, 26), we investigated if ectopic triiodothyronine (T3) alters the program of apoptosis in the GER in mice.

2. Materials and Methods

2.1. Mouse strains

Wild type C57BL/6J pups were injected (sc, at back of neck) with 1.5 μ g of T3 in a volume of 10 μ l or the equivalent volume of saline at P0 and P1. Injections were performed at 2.00 pm and cochleae were collected on the indicated days at approximately 5.00 pm. For studies of TR β -deficiency, *Thrb*^{-/-} mice originally on a mixed 129/Sv $\times$ C57BL/6J background (27) were backcrossed for 9 generations with C57BL/6J mice to create a near congenic strain on a C57BL/6J background. *Thrb*^{-/-} parents were intercrossed to generate homozygous (-/-) pups for analysis in parallel with +/+ pups of the C57BL/6J strain. Experiments were performed in accordance with guidelines at the National Institute of Diabetes and Digestive and Kidney Diseases at the National Institutes of Health.

2.2. Immunohistochemistry

Cochlear cryosections at 12 μ m thickness were fixed for 4 hours in 2% paraformaldehyde. Samples were prepared from pups between ages P1 and P13. Ages older than P13 were not

analyzed because the ossification of the cochlear bony case made it difficult to obtain cryosections that preserved adequate morphology for comparable analysis. Sections were blocked with PBS containing 1.5% goat serum, 0.1% BSA and 0.4% Triton X-100 then incubated with a 1:1000 dilution of antibody against activated caspase 3 (Promega, #G7481) overnight at room temperature. A biotinylated goat anti-rabbit secondary antibody (Vector Laboratories) was used for detection with a Vector ABC Elite kit with diaminobenzidine (Vector Laboratories). Terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) analysis was performed with the In Situ Cell Death Detection kit, according to the manufacturer's instructions (Roche). Activated caspase 3-positive cells were counted separately in apical and middle turns of the cochlea on four consecutive vertical, mid-modiolar sections on two different slides per cochlea. Counts were determined for 3 cochleas at each age examined. An indicator of the cumulative number of caspase 3⁺ cells per cochlea over developmental time was obtained by counting the sum of positive cells on representative mid-modiolar sections at P1, P3, P5, P7, P9, P11, and P13. Statistical tests were based on pairwise comparisons of a given T3-treated versus saline-treated control group using the Student's t test.

2.3. Histology

Cochleae were fixed overnight in PBS containing 3% glutaraldehyde / 2% paraformaldehyde at 4°C, dehydrated through 30, 50, 70, and 100% ethanol, then embedded in glycol methacrylate plastic (Polysciences). Mid-modiolar sections of 4 µm thickness were prepared using a microtome, then stained with aqueous hematoxylin (Biomedica Corp., Foster City, CA), as described (28). For each age, 4 to 6 cochleae from 3 mice were analyzed. Measurements of the inner sulcus area relative to the total area of the inner sulcus and GER were made on plastic sections as shown in Figure 1, using ImageJ program. Measurements were made on 7 cochlear sections from 3 saline-treated pups and 8 sections from 4 T3-treated pups.

2.4. Auditory testing

Auditory testing was performed on mice under Avertin anesthesia, as described (18). Briefly, responses were assessed in response to a click stimulus (broad band of wavelengths 1 – 16 kHz) and pure tone stimuli (8, 16 and 32 kHz) delivered with increasing intensities using an apparatus from Intelligent Hearing (Miami, Florida). Groups of 4–6 mice were tested for each treatment condition. Statistical tests were performed for a given T3-treated group versus the saline-treated group using the Student's t test.

3. Results

3.1. T3-induced remodeling and apoptosis in the GER

To determine the pattern of apoptosis during remodeling of the GER, cochlear sections were analyzed by immunohistochemistry for activated caspase 3-positive (caspase 3⁺) cells in mice between P3 and P13 following prior neonatal treatment on two consecutive days at P0 and P1 with saline or T3.

Fig. 1A shows the prematurely advanced morphology of the cochlea at P5 following this T3 treatment when examined in hematoxylin-stained plastic sections. In contrast, in saline-treated control mice, as in untreated mice (11), the organ of Corti at P5 was still immature and the GER retained a dense mass of tall columnar cells that showed only initial signs of remodeling. There was limited opening of an inner sulcus below the tectorial membrane in the area nearest to the interdental cells of the spiral limbus. The sensory epithelial region presented an immature, flattened appearance with the outer hair cells lying closely packed against the underlying Deiters' support cells. The tunnel of Corti between the pillar cells had not opened.

In T3-treated mice, the organ of Corti at P5 displayed premature remodeling with pronounced regression of the GER and an enlarged inner sulcus. The degree of opening of the inner sulcus was more than 3-fold greater in T3-treated versus saline-treated pups based on measurement of the percentage area occupied by the inner sulcus / total area occupied by the inner sulcus and GER on these histological sections. For saline-treated mice, this percentage area of the inner sulcus was 9.6 ± 2.3 % whereas for T3-treated mice, this area was 32.6 ± 0.7 % ($n = 3$ and 4 mice, respectively; $p < 0.0001$). In the sensory epithelium, the tunnel of Corti and the outer tunnel were open and the hair cells displayed an upright morphology, elevated above the Deiters' support cells. Below the opened inner sulcus, an epithelium of low cuboidal cells had formed. The residual GER in the region nearest to the sensory epithelium still contained a dense group of tall epithelial cells. In general, the advanced morphology in T3-treated mice at P5 resembled that in control mice at approximately P8 or older (11, 28). By P11 and later, remodeling in control mice had almost caught up with that of T3-treated mice and at adult ages (6–8 weeks) there was little obvious morphological difference between control and T3-treated mice (not shown).

Fig. 1B shows that at P5, caspase 3⁺ cells were not detected in cryosections of the middle turns of the cochlea in saline-treated mice but were evident in T3-treated mice. Caspase 3⁺ cells were clustered in a region of the residual GER near to the inner hair cell. Caspase 3⁺ cells were not detected in other regions of the cochlea, suggesting that T3 induced cell-specific apoptosis in a restricted region of the GER.

3.2. Developmental timecourse of apoptosis in saline and T3-treated mice

In saline-treated control mice, caspase 3⁺ cells were first detected in the GER at P7 mainly in mid-basal turns and to a lesser extent in apical turns of the cochlea (Fig. 2, shows representative middle turns). In cross-sectional view, caspase 3⁺ cells were detected in the zone of the GER nearest to the inner hair cells and distant from the initial opening of the inner sulcus.

Counts of caspase 3⁺ cells on cochlear sections were determined separately in mid-basal and apical regions to take into account the normal progression of development from base-to-apex in the cochlear duct, a general characteristic of both the morphogenesis and maturation of this spiral-shaped structure (4, 2). In middle turns, substantial numbers of caspase 3⁺ cells appeared by P7, persisted until P11, then decreased between P11 and P13 (Fig. 3). In apical turns, numbers increased later, between P7 and P9, then remained relatively high until P13. These data indicated the progression of apoptosis in a mid-basal to apical direction

consistent with the general developmental progression of the cochlear duct. As a guide to cochlear structure, Fig. 3B shows a sectional overview with the locations of basal, middle and apical turns noted.

In T3-treated mice, caspase 3⁺ cells were detected in the GER at P3, several days earlier than in saline-treated mice (Fig. 2). As in control mice, caspase 3⁺ cells were detected in the GER zone nearest to the inner hair cells. In T3-treated mice, the numbers of caspase 3⁺ cells declined in middle turns of the cochlea between P5 and P11, then later in apical turns between P7 and P13 (Fig. 3). The disappearance of caspase 3⁺ cells in T3-treated mice preceded by several days the disappearance in control mice, suggesting that T3 prematurely advanced the entire program of onset and cessation of apoptosis in the GER. The progressive appearance of apoptotic cells in the basal-to-apical direction over developmental time was similar in both saline- and T3-treated mice except that the overall program was shifted earlier by T3.

As an indicator of overall numbers of caspase 3⁺ cells detected during the remodeling of the GER, a cumulative number of caspase 3⁺ cells was counted in all turns of the cochlea in representative sections at periodic stages between P1 and P13. The cumulative number in T3-treated mice was similar to that in saline-treated mice ($p = 0.95$). T3 treatment did not result in detectable caspase 3⁺ cells in regions of the cochlea other than the GER at any developmental stage examined, suggesting that ectopic T3 acted on the same population of cells in the GER as were normally susceptible to cell death in control mice.

The findings on caspase 3⁺ cells were supported by analysis using TUNEL, an independent indicator of apoptosis, between P3 and P9. In saline-treated groups, fluorescently-labeled TUNEL⁺ cells were detected in a similar pattern as caspase 3⁺ cells beginning at ~P7 in the GER in middle turns of the cochlea. In contrast, in T3-treated mice, TUNEL⁺ cells were detected prematurely as early as P3, consistent with results from the analysis of caspase 3⁺ cells. Fig. 4B and 4D show representative data for $+/+$ mice when examined at P4: saline-treated control mice displayed almost no TUNEL⁺ cells in any turn of the cochlea whereas T3-treated mice consistently displayed TUNEL⁺ cells.

3.3. Requirement for the *Thrb* gene for T3-induced cell death in the GER

The *Thrb* gene expresses TR β receptor isoforms in the GER in the neonatal rat and mouse (29, 10) and this gene is required to facilitate morphological maturation of the organ of Corti in postnatal mice (18–20). Therefore, we tested the hypothesis that *Thrb* is required to mediate T3-induced cell death during remodeling of the GER by comparing the response to T3 in $+/+$ and TR β -deficient (*Thrb*^{-/-}) mice. Groups were treated in parallel with T3 or saline at P0 and P1, then analyzed for the presence of apoptotic cells at P4. This timepoint was selected to highlight any differences because apoptotic cells were consistently induced by ectopic T3 but not by saline in $+/+$ pups over this period (see Fig. 2).

As expected, T3 stimulated premature remodeling of the GER and sensory epithelium in $+/+$ mice at P4 (Fig. 4A shows the premature opening of the inner sulcus and tunnel of Corti in a representative mid-turn of the cochlea). T3 also stimulated the onset of apoptosis in the GER, detected as caspase 3⁺ (Fig. 4A) and TUNEL⁺ cells (Fig. 4B). In contrast, in *Thrb*^{-/-}

mice, T3 failed to stimulate remodeling of the GER or sensory epithelium, which remained in an immature state in saline- and T3-treated groups at P4. (Note the lack of inner sulcus in *Thrb*^{-/-} mice under saline- or T3 treatment). Moreover, *Thrb*^{-/-} mice were resistant to T3-induced apoptosis in the GER. Cell counts revealed that T3 consistently induced the presence of caspase 3⁺ or TUNEL⁺ cells in the GER in +/+ mice at P4 but rarely or not at all in *Thrb*^{-/-} mice under T3 treatment (Fig. 4C, 4D).

In summary, these results indicated that the *Thrb* gene is required to mediate T3- stimulated cell death in the GER during remodeling of the organ of Corti and support an underlying receptor-mediated mechanism.

3.4. Auditory function after short periods of T3 treatment

To determine if the acute T3 treatments in the neonatal period that induce premature cochlear remodeling resulted in long term defects in auditory function, the auditory-evoked brainstem response (ABR) was investigated in 6 – 8 week old mice following prior injection with T3 at P0, or on later postnatal days (Fig. 5A). Previously, we demonstrated that T3 given on four consecutive days (P0, P1, P2, P3) induced auditory defects when analyzed at P28 (28). The present study aimed to define the window of sensitivity more precisely. Saline-treated groups displayed normal ABR thresholds with normal waveforms (Fig. 5B). For a click stimulus (a broad frequency band of 1 – 16 kHz), responses were evoked at a threshold of 44.0 ± 5.4 (mean $\pm$ SEM) dB sound pressure level (dB SPL), in the normal range for mice (30)(Fig. 5C). In contrast, a single injection of T3 at P0 resulted in markedly elevated average thresholds of 89.0 ± 3.0 dB SPL ($p < 0.001$ versus saline-treated controls). Injection of T3 at P1 gave a similarly elevated threshold whereas T3 given two days later at P2 and P3, produced only a modest but not significant elevation of ABR thresholds ($p = 0.17$ versus saline-treated groups). T3 given at P4, P5 and P6 or later at P16 and P17 did not significantly change thresholds compared to saline-treated groups (Fig. 5C).

The results showed that T3 treatment at P0 or P1 resulted in a severe elevation of thresholds, although the residual waveforms that could be evoked suggested that auditory function in these mice was not completely abrogated. However, the magnitude of the residual response that could still be evoked with high intensity stimuli was considerably diminished in mice treated with T3 as neonates. For example, using a click stimulus at an intensity of 100 dB, which is above threshold for both saline and T3-treated mice, the amplitude of the waveform was 3.5 ± 0.9 μ V (mean $\pm$ SEM) in mice that had been treated with T3 at P0 compared to 12.4 ± 2.0 μ V in mice treated with saline ($p = 0.002$). Tests using pure tone stimuli (at 8, 16 and 32 kHz) that span the range of auditory sensitivity in mice, also revealed pronounced elevation of thresholds following T3 treatment at P0 or P1 (data not shown). These results indicate that the auditory system is particularly sensitive to damage from excessive T3 during a short neonatal window in mice.

4. Discussion

4.1. T3-induced apoptosis in the immature cochlea

We have delineated the timecourse of apoptosis during postnatal cochlear remodeling and have demonstrated that ectopic T3 given to neonatal mice prematurely induces the program of apoptosis and remodeling that normally precedes the onset of hearing.

Caspase 3⁺ and TUNEL⁺ cells were detected specifically in the GER in both control and T3-treated mice, suggesting that T3 triggers a cell-specific program of apoptosis in the columnar cells of the GER. The necessity for caspase 3-mediated apoptosis in cochlear remodeling is supported by the phenotype of caspase 3-deficient mice, which exhibit hearing loss with delayed regression of the GER, delayed opening of the tunnel of Corti and hair cell abnormalities at two weeks of age (31, 32). The delayed cochlear remodeling in caspase 3-deficient mice (32) resembles the similarly retarded phenotype in hypothyroid (11, 16, 17) and *Thrb* mutant (18–20) mice. We are unaware of studies of apoptosis in the cochlea in hypothyroid mice but we would expect apoptosis to be impaired, which is supported by a report that in methimazole-treated, hypothyroid rats at P7, TUNEL-staining was lacking in the GER (8).

Apoptosis in the GER is presumably mediated by the regulation of currently unknown genes by thyroid hormone receptor β which is expressed in the GER (29, 28). Caspase 3, a terminal effector of apoptosis, itself may not respond directly to T3 since caspase 3⁺ cells appear with a lag of some days after T3 promotes opening of the inner sulcus. Indeed, activation of caspases is a common endpoint of diverse signals in tissue remodeling (33, 34). It has been reported that immunoreactivity for the neurotrophin receptor p75NTR, a mediator of neuronal survival or differentiation, is detected in the GER in rats at P5 and that this immunoreactivity changed in response to thyroxine (T4) (8). However, it is unclear if p75NTR contributes to apoptosis in the GER because p75NTR-deficient mice display normal cochlear morphology and a distinct phenotype of adult-onset hearing loss with degeneration of spiral ganglion and hair cells after 4 months of age (35).

The induction of apoptosis in the GER by T3 represents a distinct cell- and temporally-specific form of apoptosis during late, post-mitotic stages of tissue maturation. This process contrasts with the more general occurrence of apoptosis that accompanies cell proliferation at earlier stages of embryogenesis, which is thought to balance cell populations during formation of many tissues (33, 25). Indeed, during proliferative stages in the embryonic rodent inner ear, caspase 3⁺ cells have been detected in the statoacoustic ganglion (31) and TUNEL⁺ cells in the statoacoustic ganglion, the cochlear duct and mesenchyme in the tympanic cavity (36). However, in the postnatal rodent cochlea, both proliferation (1) and this more general form of apoptosis (37) decline sharply before T3 induces specific cell death during remodeling of the GER.

4.2. T3-mediated apoptosis and development

The importance of developmental timing is emphasized by the finding that permanent deafness results when the immature auditory system is subject to either too much (Fig. 5) or too little thyroid hormone (11, 17). The level of T3 in the cochlea is regulated by rising

levels of T3 and T4 in the circulation between P4 and P15 in mice (38, 39) and by metabolism of the hormone by deiodination within the cochlea (10). Remarkably, ectopic T3 given only at the neonatal stage (at P0 and P1) sets in motion a chain of events that leads to cochlear remodeling over the subsequent 2 week period. Premature opening of the inner sulcus has also been noted in rats at P6 following exposure to T4 (40) and in *Dio3*^{-/-} mice lacking the thyroid hormone- inactivating type 3 deiodinase (28), although apoptosis was not investigated in these studies. The overall evidence suggests that T3 triggers the onset of apoptosis and remodeling in the GER, but that the process is completed in conjunction with other unknown signals.

Remodeling of the GER begins with retraction of the apical extensions of the columnar cells to form the inner sulcus, which occurs before apoptotic cells are detected (Fig. 1A). The columnar cells present features of autophagocytosis (7) that may reflect self-digestion of cytoplasmic contents prior to regression. Furthermore, when caspase 3⁺ apoptotic cells do appear, they are located near the hair cells somewhat distant from these first morphological changes. Thus, T3-induced remodeling may involve a sequence of pre-apoptotic events including cellular restructuring and cellular displacement before the cell death phase occurs.

The origin of the cuboidal epithelial cells that replace the columnar GER cells in the mature cochlea is unknown although these cells appear during the remodeling of the GER. One speculation is that some cells in the GER differentiate into inner sulcus epithelial cells at the same time as other columnar GER cells are eliminated by cell death. This process might resemble the T3-induced remodeling of the intestine during amphibian metamorphosis, in which larval epithelial cells are eliminated by cell death to be replaced by adult epithelial cells (41, 26).

Thyroid hormone-mediated apoptosis serves other functions in mammalian neurodevelopment. Apart from remodeling in the cochlea, thyroid hormone modifies cell death rates in the external granular cell layer of the rat cerebellum (42) and, in conjunction with nerve growth factor, determines cell survival in the rat E18 neuroblast line (43). In neonatal mice, T3 determines the survival or death of cone photoreceptors, the cells that mediate daylight and color vision (44).

Finally, our results reveal a narrow, neonatal window of susceptibility to permanent damage from excessive T3. A single injection of T3 on only one of the first two days of neonatal life (P0 or P1) results in deafness in adult mice. Although it is unclear how premature remodeling contributes to deafness, it is evident that development is precisely coordinated and must be allowed to proceed without undue haste if normal function is to be acquired. T3 provides a signal for orchestration of these events.

Acknowledgements

This work was supported by ZonMw VENI Grant: 91696017, an Erasmus MC Fellowship (RPP), and by the intramural research program at NIDDK at the National Institutes of Health.

Grants/fellowships supporting the writing of the paper:

This work was supported by ZonMw VENI Grant: 91696017, an Erasmus MC Fellowship (RPP), and by the intramural research program at NIDDK at the NIH.

Abbreviations

GER	greater epithelial ridge
TRβ	thyroid hormone receptor β

References

1. Ruben RJ. Development of the inner ear of the mouse: a radioautographic study of terminal mitoses. *Acta Otolaryngol:Suppl.* 1967; 220:221–244.
2. Wu DK, Kelley MW. Molecular mechanisms of inner ear development. *Cold Spring Harb Perspect Biol.* 2012; 4:a008409. [PubMed: 22855724]
3. Sohmer H, Freeman S. Functional development of auditory sensitivity in the fetus and neonate. *J Basic Clin Physiol Pharmacol.* 1995; 6:95–108. [PubMed: 8573562]
4. Pujol, R.; Lavigne-Rebillard, M.; Lenoir, M. Development of sensory and neural structures in the mammalian cochlea. In: Rubel, E.; Popper, A.; Fay, R., editors. *Development of the auditory system.* New York: Springer; 1997. p. 146–192.
5. Lukashkin AN, Richardson GP, Russell IJ. Multiple roles for the tectorial membrane in the active cochlea. *Hear Res.* 2010; 266:26–35. [PubMed: 19853029]
6. Rau A, Legan PK, Richardson GP. Tectorin mRNA expression is spatially and temporally restricted during mouse inner ear development. *J Comp Neurol.* 1999; 405:271–280. [PubMed: 10023815]
7. Hinojosa R. A note on development of Corti's organ. *Acta Otolaryngol.* 1977; 84:238–251. [PubMed: 906817]
8. Knipper M, Gestwa L, Ten Cate WJ, Lautermann J, Brugger H, Maier H, Zimmermann U, Rohbock K, Kopschall I, Wiechers B, Zenner HP. Distinct thyroid hormone-dependent expression of TrKB and p75NGFR in nonneuronal cells during the critical TH-dependent period of the cochlea. *J Neurobiol.* 1999; 38:338–356. [PubMed: 10022577]
9. Kamiya K, Takahashi K, Kitamura K, Momoi T, Yoshikawa Y. Mitosis and apoptosis in postnatal auditory system of the C3H/He strain. *Brain Res.* 2001; 901:296–302. [PubMed: 11368980]
10. Ng L, Kelley MW, Forrest D. Making sense with thyroid hormone-the role of T3 in auditory development. *Nat Rev Endocrinol.* 2013; 9:296–307. [PubMed: 23529044]
11. Deol MS. An experimental approach to the understanding and treatment of hereditary syndromes with congenital deafness and hypothyroidism. *J Med Genetics.* 1973; 10:235–242. [PubMed: 4774534]
12. Uziel A. Periods of sensitivity to thyroid hormone during the development of the organ of Corti. *Acta Otolaryngol (Stockh).* 1986; 101:23–27.
13. Knipper M, Richardson G, Mack A, Muller M, Goodyear R, Limberger A, Rohbock K, Kopschall I, Zenner HP, Zimmermann U. Thyroid hormone-deficient period prior to the onset of hearing is associated with reduced levels of β -tectorin protein in the tectorial membrane: implication for hearing loss. *J Biol Chem.* 2001; 276:39046–39052. [PubMed: 11489885]
14. Sprenkle PM, McGee J, Bertoni JM, Walsh EJ. Consequences of hypothyroidism on auditory system function in Tshr mutant (hyt) mice. *J Assoc Res Otolaryngol.* 2001; 2:312–329. [PubMed: 11833606]
15. Christ S, Biebel UW, Hoidis S, Friedrichsen S, Bauer K, Smolders JW. Hearing loss in athyroid Pa $\times$ 8 knockout mice and effects of thyroxine substitution. *Audiol Neuro-Otol.* 2004; 9:88–106.
16. Johnson KR, Marden CC, Ward-Bailey P, Gagnon LH, Bronson RT, Donahue LR. Congenital hypothyroidism, dwarfism, and hearing impairment caused by a missense mutation in the mouse dual oxidase 2 gene, Duox2. *Mol Endocrinol.* 2007; 21:1593–1602. [PubMed: 17440044]
17. Mustapha M, Fang Q, Gong TW, Dolan DF, Raphael Y, Camper SA, Duncan RK. Deafness and permanently reduced potassium channel gene expression and function in hypothyroid Pit1dw mutants. *J Neurosci.* 2009; 29:1212–1223. [PubMed: 19176829]

18. Rüsç A, Ng L, Goodyear R, Oliver D, Lisoukov I, Vennstrom B, Richardson G, Kelley MW, Forrest D. Retardation of cochlear maturation and impaired hair cell function caused by deletion of all known thyroid hormone receptors. *J Neurosci.* 2001; 21:9792–9800. [PubMed: 11739587]
19. Griffith AJ, Szymko YM, Kaneshige M, Quinonez RE, Kaneshige K, Heintz KA, Mastroianni MA, Kelley MW, Cheng SY. Knock-in mouse model for resistance to thyroid hormone (RTH): an RTH mutation in the thyroid hormone receptor β gene disrupts cochlear morphogenesis. *J Assoc Res Otolaryngol.* 2002; 3:279–288. [PubMed: 12382103]
20. Richter CP, Munscher A, Santana Machado D, Wondisford FE, Ortega-Carvalho TM. Complete activation of thyroid hormone receptor β by T3 is essential for normal cochlear function and morphology in mice. *Cell Physiol Biochem.* 2011; 28:997–1008. [PubMed: 22178950]
21. DeLong GR, Stanbury JB, Fierro-Benitez R. Neurological signs in congenital iodine-deficiency disorder (endemic cretinism). *Dev Med Child Neurol.* 1985; 27:317–324. [PubMed: 4018426]
22. Rovet J, Walker W, Bliss B, Buchanan L, Ehrlich R. Long-term sequelae of hearing impairment in congenital hypothyroidism. *J Pediatr.* 1996; 128:776–783. [PubMed: 8648536]
23. Brucker-Davis F, Skarulis MC, Pikus A, Ishizawa D, Mastroianni MA, Koby M, Weintraub BD. Prevalence and mechanisms of hearing loss in patients with resistance to thyroid hormone. *J Clin Endocrinol Metab.* 1996; 81:2768–2772. [PubMed: 8768826]
24. Ferrara AM, Onigata K, Ercan O, Woodhead H, Weiss RE, Refetoff S. Homozygous thyroid hormone receptor beta-gene mutations in resistance to thyroid hormone: three new cases and review of the literature. *J Clin Endocrinol Metab.* 2012; 97:1328–1336. [PubMed: 22319036]
25. Coen L, Le Blay K, Rowe I, Demeneix BA. Caspase-9 regulates apoptosis/proliferation balance during metamorphic brain remodeling in *Xenopus*. *Proc Natl Acad Sci U S A.* 2007; 104:8502–8507. [PubMed: 17488818]
26. Ishizuya-Oka A, Hasebe T, Shi YB. Apoptosis in amphibian organs during metamorphosis. *Apoptosis.* 2010; 15:350–364. [PubMed: 20238476]
27. Forrest D, Erway LC, Ng L, Altschuler R, Curran T. Thyroid hormone receptor β is essential for development of auditory function. *Nat Genet.* 1996; 13:354–357. [PubMed: 8673137]
28. Ng L, Hernandez A, He W, Ren T, Srinivas M, Ma M, Galton VA, St Germain DL, Forrest D. A protective role for type 3 deiodinase, a thyroid hormone-inactivating enzyme, in cochlear development and auditory function. *Endocrinology.* 2009; 150:1952–1960. [PubMed: 19095741]
29. Bradley DJ, Towle HC, Young WS 3rd. α and β thyroid hormone receptor (TR) gene expression during auditory neurogenesis: evidence for TR isoform-specific transcriptional regulation in vivo. *Proc Natl Acad Sci U S A.* 1994; 91:439–443. [PubMed: 8290545]
30. Zheng QY, Johnson KR, Erway LC. Assessment of hearing in 80 inbred strains of mice by ABR threshold analyses. *Hearing Res.* 1999; 130:94–107.
31. Morishita H, Makishima T, Kaneko C, Lee YS, Segil N, Takahashi K, Kuraoka A, Nakagawa T, Nabekura J, Nakayama K, Nakayama KI. Deafness due to degeneration of cochlear neurons in caspase-3-deficient mice. *Biochem Biophys Res Commun.* 2001; 284:142–149. [PubMed: 11374883]
32. Takahashi K, Kamiya K, Urase K, Suga M, Takizawa T, Mori H, Yoshikawa Y, Ichimura K, Kuida K, Momoi T. Caspase-3-deficiency induces hyperplasia of supporting cells and degeneration of sensory cells resulting in the hearing loss. *Brain Res.* 2001; 894:359–367. [PubMed: 11251216]
33. Jacobson MD, Weil M, Raff MC. Programmed cell death in animal development. *Cell.* 1997; 88:347–354. [PubMed: 9039261]
34. Yin VP, Thummel CS. Mechanisms of steroid-triggered programmed cell death in *Drosophila*. *Semin Cell Dev Biol.* 2005; 16:237–243. [PubMed: 15797834]
35. Sato T, Doi K, Taniguchi M, Yamashita T, Kubo T, Tohyama M. Progressive hearing loss in mice carrying a mutation in the p75 gene. *Brain Res.* 2006; 1091:224–234. [PubMed: 16564506]
36. Nikolic P, Jarlebark LE, Billett TE, Thorne PR. Apoptosis in the developing rat cochlea and its related structures. *Brain Res Dev Brain Res.* 2000; 119:75–83.
37. Leon Y, Sanchez-Galiano S, Gorospe I. Programmed cell death in the development of the vertebrate inner ear. *Apoptosis.* 2004; 9:255–264. [PubMed: 15258457]

38. Campos-Barros A, Amma LL, Faris JS, Shailam R, Kelley MW, Forrest D. Type 2 iodothyronine deiodinase expression in the cochlea before the onset of hearing. *Proc Natl Acad Sci U S A*. 2000; 97:1287–1292. [PubMed: 10655523]
39. Hadj-Sahraoui N, Seugnet I, Ghorbel MT, Demeneix B. Hypothyroidism prolongs mitotic activity in the post-natal mouse brain. *Neurosci Lett*. 2000; 280:79–82. [PubMed: 10686382]
40. Freeman S, Cherny L, Sohmer H. Thyroxine affects physiological and morphological development of the ear. *Hear Res*. 1996; 97:19–29. [PubMed: 8844183]
41. Brown DD, Cai L. Amphibian metamorphosis. *Dev Biol*. 2007; 306:20–33. [PubMed: 17449026]
42. Legrand J. Morphogenetic actions of thyroid hormones. *Trends Neurosci*. 1979; 2:234–236.
43. Muñoz A, Wrighton C, Seliger B, Bernal J, Beug H. Thyroid hormone receptor/c-erbA: control of commitment and differentiation in the neuronal/chromaffin progenitor line PC12. *J Cell Biol*. 1993; 121:423–438. [PubMed: 8385673]
44. Ng L, Lyubarsky A, Nikonov SS, Ma M, Srinivas M, Kefas B, St Germain DL, Hernandez A, Pugh EN Jr, Forrest D. Type 3 deiodinase, a thyroid-hormone-inactivating enzyme, controls survival and maturation of cone photoreceptors. *J Neurosci*. 2010; 30:3347–3357. [PubMed: 20203194]

HIGHLIGHTS

- The timecourse of apoptosis during postnatal remodeling of the cochlea is reported
- T3 stimulates the program of apoptosis in the greater epithelial ridge
- Evidence supports a TR β receptor-mediated mechanism of apoptosis

Figure 1. Prematurely advanced cochlear morphology (A) and appearance of caspase 3⁺ cells (B) following neonatal treatment with T3

A, Hematoxylin-stained plastic sections showing cochlear morphology (in a middle turn of the cochlea) in saline-treated control and T3-treated mice at P5. Saline or T3 was given at P0 and P1. In saline-treated mice, the organ of Corti is still immature whereas in T3-treated mice, the organ of Corti displays premature regression of the greater epithelial ridge (GER), an open inner sulcus in the region nearest the interdental cells (id) of the spiral limbus, appearance of an inner sulcus epithelium (ise) and upright morphology of the inner hair cell (ih, red triangle) and outer hair cells (oh)(grey triangles) above Deiters' support cells (dc, white triangles). The tunnel of Corti and outer tunnel (ot) are also prematurely open. Overall morphology in T3-treated mice at P5 resembles that in control mice at P8 or older.

B, Activated caspase 3-positive (caspase 3⁺) cells are not detected in cryosections in salinetreated mice but are prominent in T3-treated mice in the GER (red arrow)(middle turn of the cochlea shown). Caspase 3⁺ cells tend to cluster in an area near the inner hair cell. Groups of 3 mice were examined. Scale bar = 20 μ m.

Figure 2. Developmental appearance of caspase 3⁺ cells in the cochlea following neonatal treatment with T3 or saline

In saline-treated mice, caspase 3⁺ cells (red arrows) are first detected in the GER at ~P7 and persist at least until P9 in middle turns of the cochlea. In T3-treated mice, caspase 3⁺ cells are detected earlier, at P3, but are no longer detected after P9 in middle turns of the cochlea. T3 or saline was given on P0 and P1, then samples were analyzed at the indicated later ages. Under these conditions, the acellular tectorial membrane is prone to detachment and also gives nonspecific signals. Scale bar = 50 μ m.

Figure 3. Caspase 3⁺ cell counts during remodeling of the GER in T3- and saline-treated mice
A, In control saline-treated mice, caspase 3⁺ cell numbers peak first in mid-basal turns then later in apical turns, indicating progression of apoptosis from base to apex along the cochlear axis during development (white circle symbols, solid line). T3-treatment prematurely increases caspase 3⁺ cell numbers in both middle and apical turns of the cochlea (black triangle symbols, dashed line). A similar gradient occurs along the base-apex axis as in control mice: the peak in middle turns precedes that in the apex. T3 also causes the premature developmental disappearance of caspase 3⁺ cells in both middle and apical turns.

Cell counts were determined in the organ of Corti in middle and apical turns of the cochlea on 10 μm thick cryosections.

B, Vertical overview section of the cochlea (at 6 weeks of age) shows the organ of Corti (oc) in basal, middle and apical turns. Hematoxylin-stained plastic section. M, modiolus; oc, organ of Corti.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 4. Requirement for the *Thrb* gene for T3-induced apoptosis in the organ of Corti

A, Immunostaining for caspase 3⁺ cells in representative mid-turn sections of the cochlea at P4 following treatment with saline or T3 at P0 and P1. T3 stimulated premature opening of the inner sulcus, formation of the inner sulcus epithelium (ise) and opening of the tunnel of Corti (t). T3 induced the presence of caspase 3⁺ cells (red arrows) in the GER in +/+ but not *Thrb*^{-/-} mice. Inner and outer rows of hair cells are indicated by black-filled and white-filled triangles, respectively. The tectorial membrane (tm) stains non-specifically.

B, Immunofluorescence analysis for TUNEL in the same groups as in panel A. TUNEL⁺ cells were induced by T3 in +/+ but not *Thrb*^{-/-} mice.

C, Counts of caspase 3⁺ and TUNEL⁺ cells from the same groups as in panels A and B. Counts were determined separately in basal, middle and apical turns of the cochlea in 10 μ m thick cryosections; n = 4 sections per cochlea from 6 cochleae from 3 mice per group (**, p < 0.0001 for T3- versus saline-treated +/+ groups in apex and mid-regions, *, p < 0.05 for same comparison in basal region).

Figure 5. Auditory function in mice following transient, short-term treatment with T3 during postnatal development

A, Experimental scheme. Groups of mice were treated with saline or T3 on the postnatal days shown then tested for auditory-evoked brainstem responses (ABR) at 6 – 8 weeks of age. Groups contained 4 – 6 mice.

B, Representative families of ABR waveforms in response to a click stimulus at different intensities (dB SPL) in individual mice treated with either T3 at P0 or saline at P0 and P1. The saline-treated mouse displays a normal pattern of peaks within 4 – 5 ms of the stimulus

but the T3-treated mouse displays identifiable peaks only in response to high intensity stimuli. For the saline- and T3-treated mice shown, thresholds were 45 and 95 dB SPL, respectively (underlined). Waveforms are shown on a normalized scale to optimize detection of the smaller peaks in the T3-treated mice. For the waveforms shown, maximal amplitudes are 9.89 μ V at 70 dB SPL for the saline-treated mouse and 5.17 μ V at 100 dB SPL for the T3-treated mouse.

C, Mean ABR thresholds ($\pm$ SEM) for groups treated at different postnatal stages. A single injection of T3 at P0 or P1 results in markedly elevated average thresholds whereas T3 given at P2 and P3, or later stages, produces only modest elevation of ABR thresholds. ***, $p < 0.001$ for T3-treated group versus saline treated control group.