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Characterization of Deiodinase Expression in the Hypothyroid Developing Cochlea

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By

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This thesis has been examined and approved by the following members of the student's committee.

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Abstract
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Thyroid hormone (TH) is essential for cochlear development and normal auditory function. Considering the importance of TH in mediating cochlear development, understanding the degree to which developing tissues can adapt to perturbations in thyroid hormone signaling is extremely important. The deiodinases (D2 and D3) are enzymes that tightly control TH availability at the tissue level and have been proposed to function as adaptive mechanisms that maintain tissue TH homeostasis. D2 converts thyroxine (T4) to a biologically active ligand triiodothyronine (T3); locally amplifying a T3 signal. Conversely, Dio3 inactivates T3 and T4 by converting these iodothyronines to the inactive metabolites diiodothyronine (T2) and reverse T3 (rT3), respectively. During cochlear development, Dio3 expression is high prenatally while Dio2 expression is low. During the first postnatal week, the expression levels of these enzymes invert, resulting in high expression of Dio2 and low expression of Dio3. Together, the deiodinases control the timing of postnatal cochlear remodeling; suggesting a genetic developmental clock controls TH-mediated cochlear development.

Considering the role deiodinases have as an adaptive mechanism, it is important to understand whether such a developmental clock can be negated during times of developmental thyroid hormone insufficiency. We hypothesize that the perinatal change in deiodinase expression is controlled by a developmental clock rather than environmental clues and therefore have limited capacity to function as compensatory mechanisms in response to low TH during development. To test this, timed-pregnant mice were treated with thyroid gland inhibitors to induced hypothyroidism from gestational day 12.5 until pup sacrifice. A parallel set of untreated timed-pregnant mice served as controls. Cochlea were harvest from control and hypothyroid mice at postnatal ages P1, P5, P10 and P15 for qRT-PCR. We observed D2 and D3 mRNA levels were similar or reduced in hypothyroid animals compared to controls at P1, P5, and P10. This finding indicates that at these ages, D2 at did not respond in a manner consistent with the idea of compensation. However, at P15, D2 mRNA levels were increased in hypothyroid animals compared to controls; a finding that is consistent with a compensatory mechanism. Interestingly, decreased D3 mRNA levels observed in the hypothyroid cochlea is indicative of an adaptive response that could be attempting to compensate for the goitrogen-induced reductions in T4. Taken together, our results suggest that developmental programs in tissues may be dominant over potential compensatory mechanisms and that developing tissues may be more susceptible to perturbations in tissue TH levels due to a reduced capacity to compensate.

Introduction

Thyroid hormone is essential for proper development of the nervous and sensory systems (Sharlin 2015). Particularly, development of the cochlea requires active thyroid hormone (triiodothyronine; T3) for cochlear differentiation and ultimately proper auditory system function. Thyroid hormone must be available during critical developmental windows prior to the onset of auditory function because at later stages auditory deficits cannot be reversed by thyroid hormone supplementation (Ng et al. 2004; Sharlin 2016).

Deiodination reactions carried out by Type 2 deiodinase (D2) and Type 3 deiodinase (D3) regulate thyroid hormone signaling at the tissue level due to their activating and deactivating properties, respectively (Williams and Bassett 2011). In many developing tissues, including the cochlea, pre- and perinatally D3 activity is high where as D2 activity levels are low. Postally, the activity pattern inverts, with D3 levels low and D2 levels high. This pattern is known as the "double switch" and is required to trigger remodeling of cochlear tissues and timing for the onset of auditory function. Previous research has demonstrated that in the absence of D2-mediated amplification of a T3 signal during the early postnatal period (resulting in a local tissue hypothyroidism), development events of the cochlea are delayed that results in the improper formation of critical structures for the onset of hearing (Ng et al. 2017, 2009).

Based on these observations, we hypothesize that the perinatal changes in deiodinase expression in a hypothyroid individual will follow the delayed onset of

important remodeling events in the cochlea because this switch is controlled by a developmental clock rather than environmental clues and therefore have limited capacity to function as compensatory mechanisms in response to low TH during development.

Literature review

HPT Axis

The synthesis and secretion of thyroid hormone, and thus circulating levels, is mainly regulated through negative feedback of the hypothalamus-pituitary-thyroid axis (HPT axis). The hypothalamus communicates through thyrotropin releasing hormone (TRH) neurons that originate in the paraventricular nucleus. (Sharlin 2015; Ng et al. 2013; Santisteban 2013). These neurons release TRH to the anterior pituitary and stimulate thyrotrope cells to secrete thyroid stimulating hormone (TSH) into the bloodstream. TSH regulates the activity of the thyroid gland, which secretes thyroxine (T4) and triiodothyronine (T3) directly into the bloodstream. The thyroid gland secretes higher amounts of the prohormone T4 than the active form T3. T4 is converted into the biologically active form T3 locally in tissues by extrathyroidal deiodination enzymes; providing a mechanism to regulate TH action directly in a tissue.

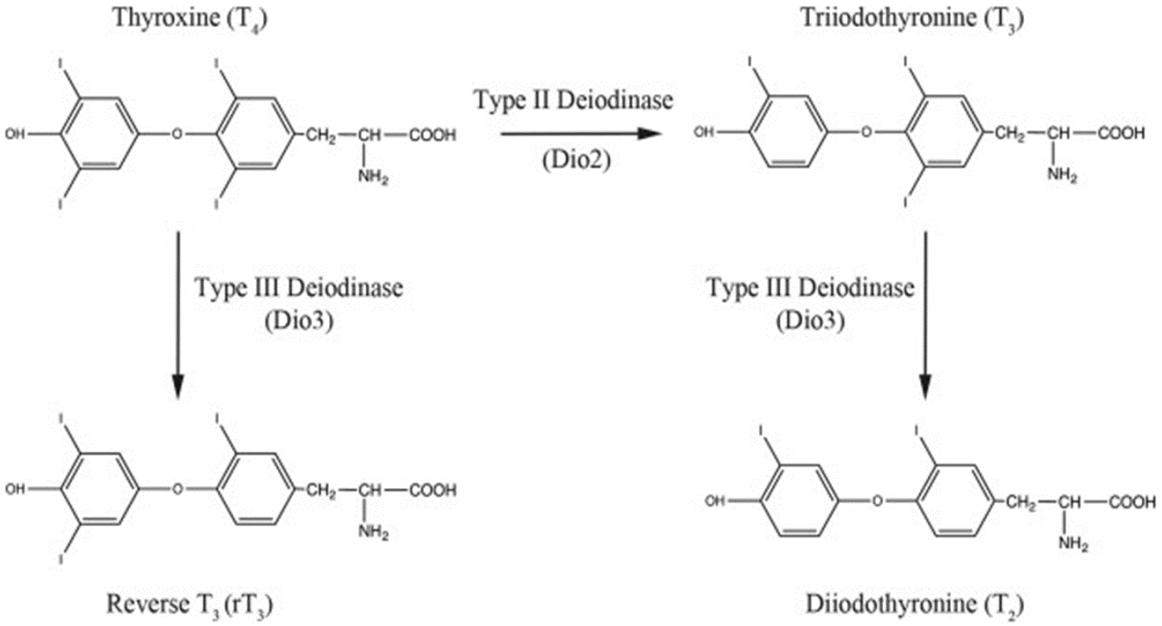

Figure 1. Overview of the deiodination process of thyroxine (T4) into Triiodothyronine (T3) by Type II deiodinase enzyme or Reverse T3 (rT3) by type III deiodinase enzyme. Picture taken from Bianco et al, 2011.

Deiodinases

Iodothyronine deiodinases are peroxidase enzymes that regulate the local extra- and intracellular concentrations of thyroid hormone thereby regulating thyroid hormone action at receptors (Williams and Bassett 2011). As mentioned above, thyroid hormone is secreted into the blood stream as T3 and T4, with T4 secretion being 10 times greater than T3. T4 must be converted to T3 by deiodination of an iodine from the outer phenolic ring to yield the biologically active T3 (Bianco and Kim 2013). The deiodination reaction is carried out by three different types of deiodinases. Type 1 deiodinase can catalyze both the activating and inactivating reactions. This type is mainly found in the liver and is proposed to be

important in metabolizing thyroid hormone for excretion. D2 catalyze the activating reaction; while D3 has a role in the inactivation of both T3 and T4 (Fig. 1). In the cochlea, Type 1 is not present. However, important roles for D2 and D3 enzymes have been reported (Ng et al. 2017, 2009).

The expression of both D2 and D3 enzymes is regulated in a tissue- and time-specific manners, which as a result controls the varying levels of T3 action at different times during development. In the developing cochlea, D3 is high during embryonic and perinatal timepoints when D2 levels are low. This high level of D3 in tissues T3 action at the receptor. Postnatally, the expression pattern inverts; with D3 decreasing to low levels during the first postnatal week while there is a simultaneous and robust increase in D2 levels until about postnatal day 10 at which times D2 levels begin to fall to adult levels. Such developmental and robust changes in deiodinase are likely due to histone modifications known to mediate changes in gene expression by modifying the chromatin structure and freeing compact chromatin into an accessible form for transcription factors to bind, enhancing gene expression (Ambrosio et al. 2013). This idea is supported by the findings that in muscle histone H3 demethylating enzyme (LSD-1) is necessary for induction of D2 transcription and repression of D3 in muscle pre-cursor cells. (Ambrosio et al. 2013). It is unknown whether similar histone modifying mechanisms operate in the developing cochlea.

Thyroid hormone receptors

Encoded by two independent genes, *thra* and *thrb*, thyroid hormone receptors (TR) serve as ligand-regulated transcription factors that mediate specific actions of T3 (Ng et al.2013). Alternative splicing of the *thra and thrb* genes generates receptor subtypes TRα1, TRβ1, TRα2 and TRβ2 (Jones et al. 2003). All four isoforms are expressed in the cochlea (Bradley et al. 1992). TRα1, TRβ1, and TRβ2 are bona fide receptors that bind T3. In contrast, the TRα2 isoform lacks a ligand binding domain and thus can bind DNA, but since it is unable to bind ligand, it is unable mediate ligand-dependent TH action.

In the cochlea, TRα transcripts are mostly found prenatally in the sensory epithelium, spiral ganglion and the vestibular system, suggesting that these regions are sensitive to circulating T3 levels before birth and any disruptions may potentially lead to pathophysiological changes (Lautermann and Ten Cate 1997). However, mice lacking TRα do not show auditory impairment (Rusch et al. 2001). TRα2 has been found to be expressed in the same regions as TRα1 in the cochlea (Sharlin 2016; Bradley et al. 1992).

Additional knockout studies demonstrated TRβ is essential for cochlear development and auditory function (Rusch et al. 2001; Ng et al. 2001). TRβ1 and TRβ2 are expressed in several cochlear regions, including the greater epithelial ridge (GER) and sensory epithelium. TRβ expression overlaps TRα in the GER and sensory epithelium. TRα1 is expressed at lower levels than TRβ1 and TRβ2 (Cordas et al. 2012). Interestingly, the TRβ knockout mice are deaf (Rusch et al. 2001; Lily Ng et al. 2001; Griffith et al. 2002)

while the TRα knockout mouse has normal hearing abilities (Rusch et al. 2001; Ng et al. 2001). Double knockouts of both TRβ and TRα exhibit aggravated deficits in hearing when compared to the single knockouts of TRβ. This difference in hearing deficits suggests that TRα is highly involved in the development of hearing (Richter et al. 2011; Winter et al. 2007; David J. Bradley, Towle, and Young 1994; Rusch et al. 2001).

Thyroid hormone in Cochlear development

Physical and mental disabilities have been observed in individuals with congenital thyroid hormone disorders such as congenital hypothyroidism or resistance to thyroid hormone due to mutations in the receptor genes (Rusch et al. 2001; Sharlin 2015; Peeters et al. 2015). These observations highlight the importance of thyroid hormone signaling during specific developmental stages of the nervous system. In particular, deafness caused by improper development of the cochlea, the auditory sensory organ, has been identified as a TH target tissue in developmental thyroid hormone insufficiency in both humans and rodent models (Ng et al. 2017).

Experimental studies have demonstrated that cochlear development is sensitive to T3 towards the end stages of cochlear differentiation. In humans, this period occurs between 9 and 18 weeks of gestation (Locher et al. 2015). In rodents, the overall structure of the cochlea is formed before birth but continues to remodel until around 2 weeks of age (Peeters et al. 2015). Specifically, T3 influences the remodeling of the GER, a transient structure that regresses during the first two postnatal weeks. This mass of cells gives way

to the inner sulcus and secretes the proteins that will form the acellular tectorial membrane, a critical structure for auditory function (Sharlin 2016; Peeters et al. 2015).

Additionally, hypothyroid models have shown that decreased exposure to T3 during development causes a delay in important physiological functions in inner and outer hair cells. Additionally, insufficient TH has also be reported to delay the remodeling GER and inner sulcus epithelium. This disruption results in improper suspension of the tectorial membrane, as well as improper expression of membrane glycoproteins α and β-tectorin. This observation provides a possible explanation for the swelling and disorganized microstructure of the tectorial membrane observed in hypothyroid cochlea (Knipper et al. 2001). Similarly, experiments in mice lacking thyroid hormone receptors have defined an essential role for TRβ in GER regression and tectorial membrane formation (Rusch et al. 2001).

Fewer studies have examined the effects of hyperthyroidism on cochlear development, but the general observation is that effect is opposite to the effects observed in hypothyroid models. Ng et al. (2009) injected excessive T3 into neonatal mice and measured their auditory function 28 days after injections. This study tested the auditory evoked brainstem response (ABR) and the distortion product otoacoustic emission (DPOAE) which showed that excessive exposure to T3 caused severe auditory deficits (Ng et al. 2009).

Role of Deiodinases in Cochlear Development

D2 is expressed in the connective tissue surrounding the sensory epithelium and its activity peaks 7 days after birth (P7). D2 has an essential role in the availability of active thyroid hormone in the developing cochlear tissue and therefore is necessary for the proper formation of the cochlea and normal auditory function (Williams and Bassett 2011). In the D2 knockout mouse, GER remodeling is delayed and auditory function is deficient, similar to deficiencies found in individuals with complete absence of receptors as well as those with severe hypothyroidism (Ng et al. 2004; 2009). These phenotypic findings highlight the importance of proper D2 expression during the developmental stages.

Just as crucial, D3 plays a role in the proper development of the cochlea. D3 diminishes the actions of T3 and T4 in the tissue by producing biologically inactive reverse T3 (rT3) or diiodothyronine (T2). Studies with D3 knockout mice showed that the primary role of this enzyme is to protect the GER from premature exposure to thyroid hormone. In its absence, there is early differentiation of the GER which leads to poor auditory function (Ng et al. 2009). The same study also showed that the expression of this enzyme is, for the most part, limited to the GER and the stria vascularis.

Adaptive mechanism to maintain normal tissue TH action

The HPT controls circulating levels of thyroid hormone within a relatively narrow range. Hypothyroidism (under-active thyroid gland) is a thyroid condition characterized by low circulating thyroid hormone levels in the blood. Hyperthyroidism (over-active thyroid

gland), is a condition characterized by excess thyroid hormone in circulation. Having either of these disorders can be unfavorable, especially for an organism with developing tissues. It is proposed that developing and mature tissues possess adaptive mechanisms that aim to normalize tissue thyroid hormone levels in situations where disorders in serum thyroid hormone occur (Sharlin et al. 2010). One specific adaptive mechanism is the deiodinases, which may protect developing tissues due to their ability to activate or inactivate thyroid hormone locally. Therefore, the direct availability of this hormone is dependent on the upregulation or repression of these enzymes at a tissue-specific level. For example, D2 is sharply upregulated during myoblast differentiation whereas D3 is downregulated (Ambrosio et al. 2013). Particularly in the cochlea, studies in both D2 and D3 knockout mice have demonstrated the importance of deiodinases in the proper timing of thyroid hormone action in the developing cochlear tissue (Ng et al. 2009, 2017).

From the above information it can be concluded that development of the cochlea and normal auditory function requires a tightly controlled and regulated by a time dependent availability of T3. This is achieved by synchronized and developmentally inverted expression and activity of D2 and D3. Prenatal D3 activity is maintained at high levels, with low D2 activity levels. Later, after birth, the activity pattern of these enzymes changes, with increasing D2 activity as D3 activity is reduced, allowing T3 to trigger remodeling of the tissues and the onset of auditory function. However, it is unknown whether the expression of these enzymes change in response to altered thyroid hormone

levels in a manner that would be consistent to these enzymes functioning as adaptive mechanisms aimed at preventing tissue malformation.

The tissue activities of D2 and D3 change in response to changes in circulating TH. During a hypothyroid state, studies have demonstrated an increased expression of D2 in the brain (Guadaño-Ferraz et al. 1999), which could represent a compensatory response to the low levels of T3 because increased D2 activity will increase the conversion rate of T4 to T3 to provide adequate support to the T3. However, whether the same increase in D2 happens in the T3 responsive cochlear tissues is still unknown. Simultaneously, D3 activity decreases in the hypothyroid organism which allows for a longer residency of T3 around the tissue (Bianco and Kim 2013).

This thesis investigates whether cochlear abnormalities following developmental hypothyroidism are amplified by a delay in the deiodinase switch due to an overall delay in tissue maturation. We hypothesize that in hypothyroid mice, the normal postnatal decrease in Dio3 expression and concomitant increase in Dio2 will be delayed compared to euthyroid mice. If observed, we propose that these altered expression levels are due, in part, to the overall delay in cochlear development know to occur with developmental hypothyroidism. Additionally, such an observation would suggest that developmental programs that mediate tissue development are dominant over potential adaptive mechanisms that aim to maintain tissue TH action; preventing compensation from occurring.

Materials and Methods

Animals

C57BL/6J mice from Jackson Laboratory were housed in the animal care facility in Minnesota State University, Mankato. Mice were given LabDiet 5001 Rodent Diet chow and water *ad libitum*. All procedures were approved by Minnesota State University, Mankato's Institutional Animal Care and Use Committee (IACUC). Breeding dams were monitored for the presence of sperm plug daily with a positive plug considered embryonic day 0.5 ((E)0.5).

Hypothyroid Treatment

On embryonic day (E)12.5 the drinking water of selected cages was replaced with water containing with 0.02% methimazole **(**MMI; inhibits thyroid gland thyroperoxidase activity) and 1% $KClO_4$ (inhibits the sodium iodide symporter). Treatment was monitored daily and replaced every 2-3 days. Treatment of pregnant dams continued through the embryonic period and continued postnatally until all the test subjects in the cage were sacrificed. Euthyroid animals received untreated water from the facility and used as control.

Sacrifice and Sample Collection

Mice were sacrificed at post-natal day (P) 1, P5, P10 and P15. At the two early ages, pups were rapidly decapitated. At the later ages, pups were euthanized by placement in a CO_2 chamber. Following euthanasia, both cochleae were dissected from each animal using a dissecting scope. Cochleae used for qPCR were flash frozen by placing them in dry ice and stored at -80°C until RNA purification. Cochleae used for *In-situ* hybridization were fixed in 4% paraformaldehyde (PFA) in 1xPBS for 3 hours at 4°C with light rocking. Cochlea were then washed (3x10 minutes) with 1xPBS and then placed in a 100mM ethylenediaminetetraacetic acid (EDTA)/ 1xPBS/30% sucrose solution for 24 hours at 4°C with light rocking. The following day, samples were embedded in optimum cutting temperature medium (OCT; Tissue-tek), frozen on dry ice and stored at -80°C until cryosectioning. Additionally, a tail sample was collected for sex genotyping from P1 and P5 pups. Trunk serum was also collected following euthanasia from both the dam and pups to later perform a T4 ELISA for verification of euthyroid or hypothyroid state. After collection, whole blood was placed on ice for no longer than 30 mins and then centrifuged at 12000rpm for 10 minutes. After centrifugation, serum was removed from the tube and stored at -20°C until further use.

Cryosectioning

Mid-modiolar cochlear sections were cut at 16 μm. Sections were thaw mounted onto Superfrost Plus/Colorfast Plus Microslides (75x25mm; Daigger) and stored at -80°C until staining for *in situ* hybridization.

SRY Genotyping

P1 and P5 tails were lysed in 500µL of tail lysis buffer (10 mM Tris, pH 8.0, 100 mM NaCl, 10 mM EDTA, pH 8.0, 0.5 %SDS) with 20µg/mL Proteinase K. Samples were incubated at 56°C with constant shaking at 1500rpm for 2 hrs. After lysis, samples were centrifuged for 10 minutes at 13200rpm to remove debris and 450µL of the supernatant was removed from each sample avoiding the pellet. Then, 450 µL of isopropanol was added to each tube containing the supernatant. Next, the samples were mixed by inverting to allow DNA precipitation. Precipitated DNA was transferred into 500µL of DEPC-H_2O and shaken at 1000rpm for 5 minutes at 56°C to dissolve DNA.

To detect the SRY gene by PCR, 0.2 µM forward and reverse SRY primers and 0.12µM forward and reverse primers for IL3, nuclease free H_2O, and 2x GoTaq PCR Master mix (Promega) were mixed to a final volume of 19ul. 1µL of each DNA sample was added. Samples were then mixed, briefly centrifuged, and placed in a thermal cycler as follows: 95°C for 4 minutes, followed by 35 cycles of 95°C for 30 seconds, 50°C for 1 minute, and 72°C for 1 minute. Then, the samples were incubated at 72°C for 6 minutes and held at 4°C. All samples were electrophorosed on a 1.5 % agarose gel with one band indicating females and two bands indicating males (Fig. 2).

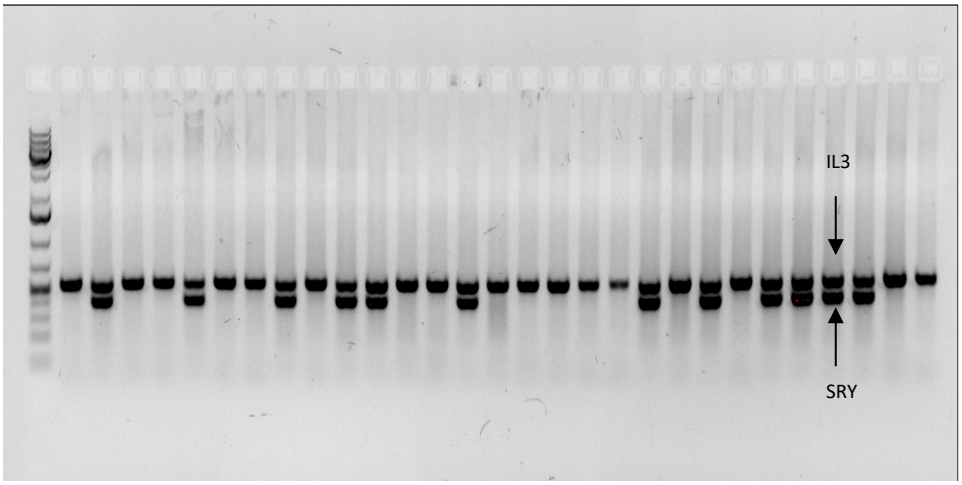

Figure 2. SRY genotyping. All lanes show a band for control gene, IL3. Males have a second band for male-specific gene SRY.

ELISA Assay

Concentrations of T4 in serum were measured by Enzyme-Linked Immunosorbent Assay (ELISA) using the AccuDiag™ T4 ELISA Kit and the manufacturer's protocol (Diagnostic Automation/Cortez Diagnostics Incorporated).

RNA isolation and cDNA synthesis

Total RNA was isolated using the RNeasy Plus Universal Mini kit (Qiagen). The concentration and purity of the isolated RNA was measured using a nanodrop and stored at -80°C. RNA quality was determined by gel electrophoresis of 2 µL of each sample on a 1% agarose bleach gel. Pure samples showed two distinct bands: a larger 28S rRNA band, a smaller 18S rRNA band and a lack of genomic DNA band contained in the gel well (Fig 3).

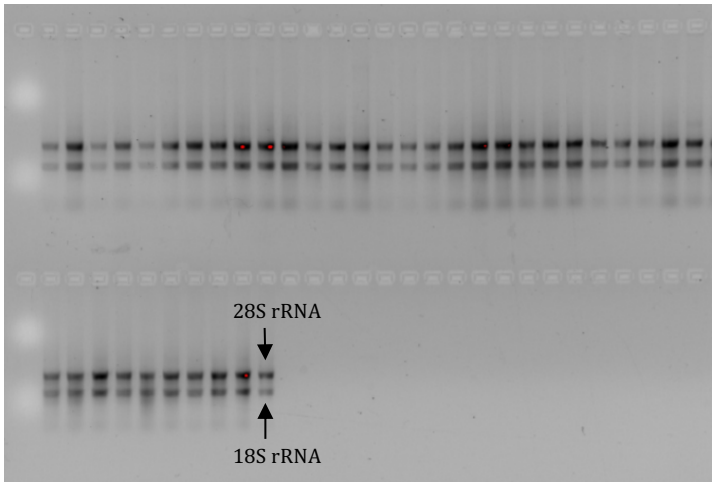

Figure 3. Bleach gel for RNA quality. RNA was of good quality. Indicated by the presence of two bands: 28s rRNA and 18s rRNA.

cDNA was synthesized from total RNA using the SuperScript IV VILO Master Mix (Thermo Fisher). Samples with a concentration below 90 ng/µL used 200 ng total RNA as a template. All other samples used 500 ng of total RNA as a template. Following cDNA synthesis, sample reactions were diluted to a final RNA mass of 50ng/µL.

Real-Time Quantitative PCR (mRNA RT-qPCR)

Using TaqMan Gene expression Assay probes (ThermoFisher; Table 1), D2 and D3 mRNA transcripts were quantified using a Step One Plus Real-Time PCR machine (Applied Biosystems). The relative amounts of RNA expression were calculated using 18S rRNA as an internal normalization control. Relative RNA amount was determined by the standard curve method using 5-fold dilution. RNase-free water was used as a negative template

control in the place of a cDNA template. The thermocycler program followed the TaqMan probe protocol. The efficiency of each primer set was calculated. For the amplification, the thermocycler program includes the following steps: initial denaturation at 95°C for 10 minutes, 40 cycles at 95°C for 15 seconds each, and an annealing/extension step at 60°C for 1 minute

Table 1. qRT-PCR primers (Applied Biosystems)

Gene	Primer-Probe Reference Number
dio2	Mm00515664_m1
dio3	Mm00548953_s1
18S rRNA	Mm03928990_g1

In Situ Hybridization: Probe Generation

Dio3 cDNA cloning

To clone a Dio3 cDNA fragment (NM_172119.2) primers were tested to determine proper concentration and annealing/extension temperature for optimal efficiency. Two primer sets (Table 2) were tested (a and b) at three different concentrations (0.1 µM, 0.3 µM, 0.5 µM) and temperatures (68 °C, 65 °C, 60 °C). Samples were diluted and placed in thermocycler with the following protocol: 95°C for 2 minutes; 40 cycles of 95°C for 15 seconds, 60-68°C for 15 seconds, 72°C for 5 minutes; infinite hold at 4°C. Samples were

electrophorosed on a 1.5 % agarose gel. The primer selected was set A at a 0.3 µM with an annealing temperature of 60°C because it showed a clear band without any smearing as shown in fig. 4. This primer set amplifies base pairs 329-865 of *Mus musculus* Dio3 mRNA (NM_172119.2).

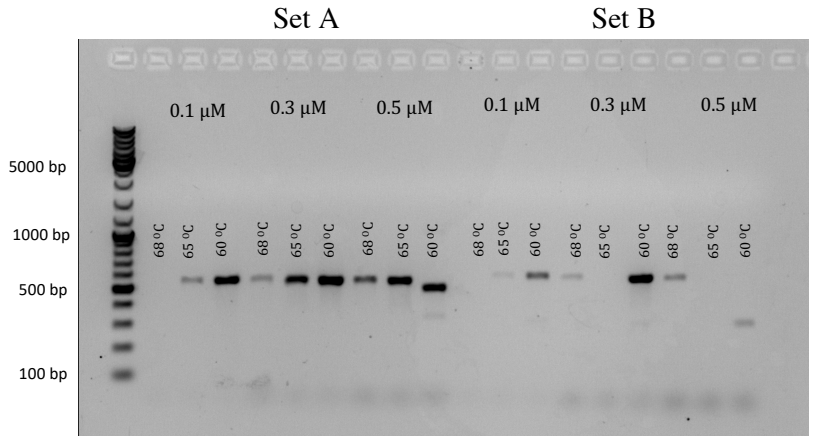

Figure 4. Dio3 partial cDNA primer test. Primer sets A and B were tested at concentrations 0.1 µM, 0.3 µM, 0.5 µM and temperatures 68 °C, 65 °C, 60 °C. The primer selected was the one with the clearest band and minimal smearing: A,0.3 µM, 60°C.

Table 2. Partial Dio3 cDNA primers.

Primer Set	Forward Primer	Reverse Primer	Amplicon size	Base pairs	Accession #
A	5' -CACCCTGGCCTCTCTCAAAG-3'	5' -CAAGTGCGCAACTCAGACAC-3'	536 bp	327-863	NM_172119.2
B	5' -GTGGTCGGAGAAGGTGAAGG-3'	5' -GGTGGGCTTCCTCGATGTAG-3'	579bp	28-607	NM_172119.2

Ligation

Amplified fragments obtained from PCR described above was purified using GeneJET Gel Extraction and DNA Cleanup Micro Kit protocol (Thermo Fisher). Ligation of partial Dio3 cDNA and pGEMT-Easy vector was performed using pGEM T-Easy Vector (Promega), 2x rapid ligation buffer, T4 DNA Ligase, and purified PCR product. Purified plasmids were digested to verify that they contained the Dio3 insert (Fig. 5).

Transformation

JM109 cells were transformed with a Dio2 and Dio3 plasmids and plated on ampicillin/IPTG/X-Gal-containing agar plates. Several single colonies were selected to grow in culture of luria broth (LB) with ampicillin overnight.

DNA Plasmid Purification

Genejet Plasmid Miniprep kit protocol (Thermo Fisher) was used to purify the plasmid following manufacturer instructions. Concentration of purified plasmid was determined spectroscopically using a nanodrop and purified plasmids were stored at -20°C.

Plasmid Digest

EcoR1 enzyme (New England Biolabs) was used to verify proper insert size. The samples were electrophoresed on agarose gel. Colony 9 was selected for sequencing. Sequencing confirmed the proper Dio3 cDNA insert (data not shown).

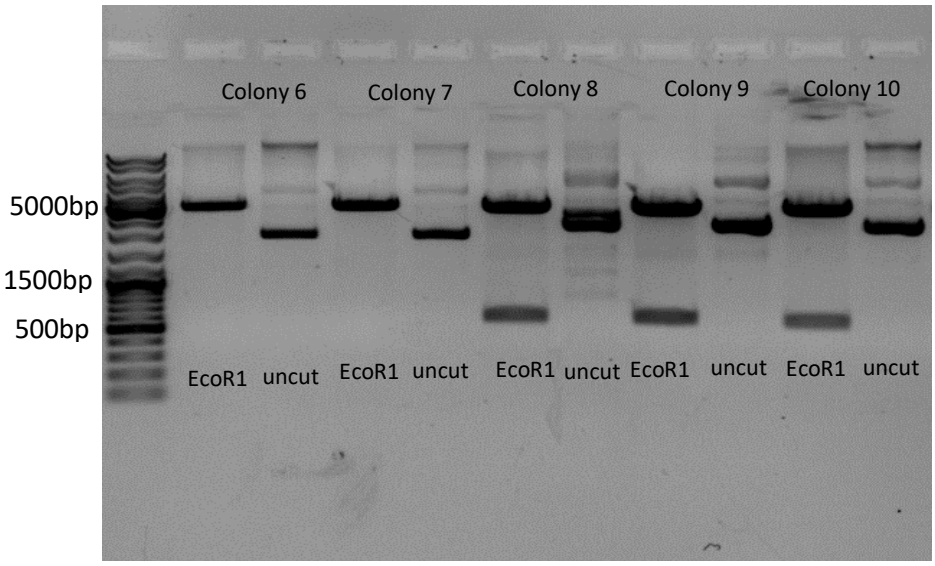

Figure 5. Dio3 plasmid digest. EcoR1 restriction endonuclease was used to cut the plasmid. The cut sample was sent for sequencing to verify the presence of Dio3 insert. EcoR1 resulted in 600bp and 5000 bp fragments shown on the gel. Colonies 9 and 10 were selected because they showed high concentration and fragments of appropriate lengths.

Plasmid Linearization

Deiodinase plasmid was linearized using two single-cutter enzymes to obtain sense and anti-sense single strands (Fig. 6). For Dio2, Hind III enzyme was used to cut 0.5 µg of plasmid to obtain the anti-sense probe and PST I enzyme (New England Biolabs) was used to cut 0.5 µg of plasmid for a sense (negative control). For Dio3, Sac II was used to cut 0.5 µg of plasmid for anti-sense probe and PST I enzyme was used to cut 0.5 µg of plasmid for a sense probe (Table 3). The plasmid was incubated with the restriction enzymes for 2 hours then purified using GeneJET Gel Extraction and DNA Cleanup Micro Kit protocol

(Thermo Fisher). The concentration of the purified, linearized DNA template was determined, and samples were electrophorosed on a gel to confirm proper linearization.

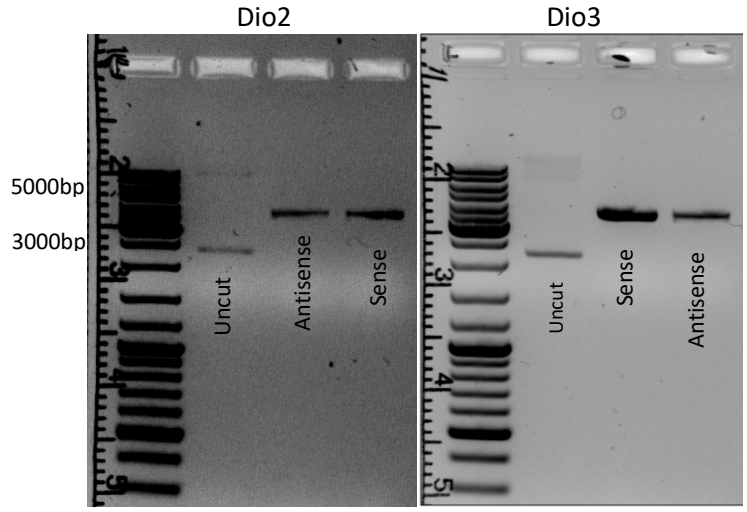

Figure 6. Dio2 and Dio3 linearized sense and antisense plasmids. Linearized plasmids obtained by using restriction endonucleases. Linearized plasmids were approximately 4800bps

Table 3. Single-cutter enzymes used obtain sense and anti-sense single strands (New England Biolabs)

Plasmid	Antisense Enzyme	Sense Enzyme
Dio2	HindIII	PST1
Dio3	NcoI	SalI

In vitro Transcription of RNA Probes.

The DNA templates were transcribed using RNA polymerase enzymes: for Dio2, T3 for anti-sense and T7 for sense. For Dio3, Sp6 for anti-sense and T7 for sense (Promega) (Table 3; Fig. 7). The plasmid DNA templates were incubated in a 50µL reaction with 5 µL RNA polymerase, 5 µL of 5x transcription buffer (Promega), 5 µL 10 mM digoxygenin NTP mix (Roche), 1 µL RNAse inhibitor (Promega), and nuclease free water at 37 °C for 2 hours. DNase I (Roche) was then added and incubated at 37 °C for 1hr to remove the

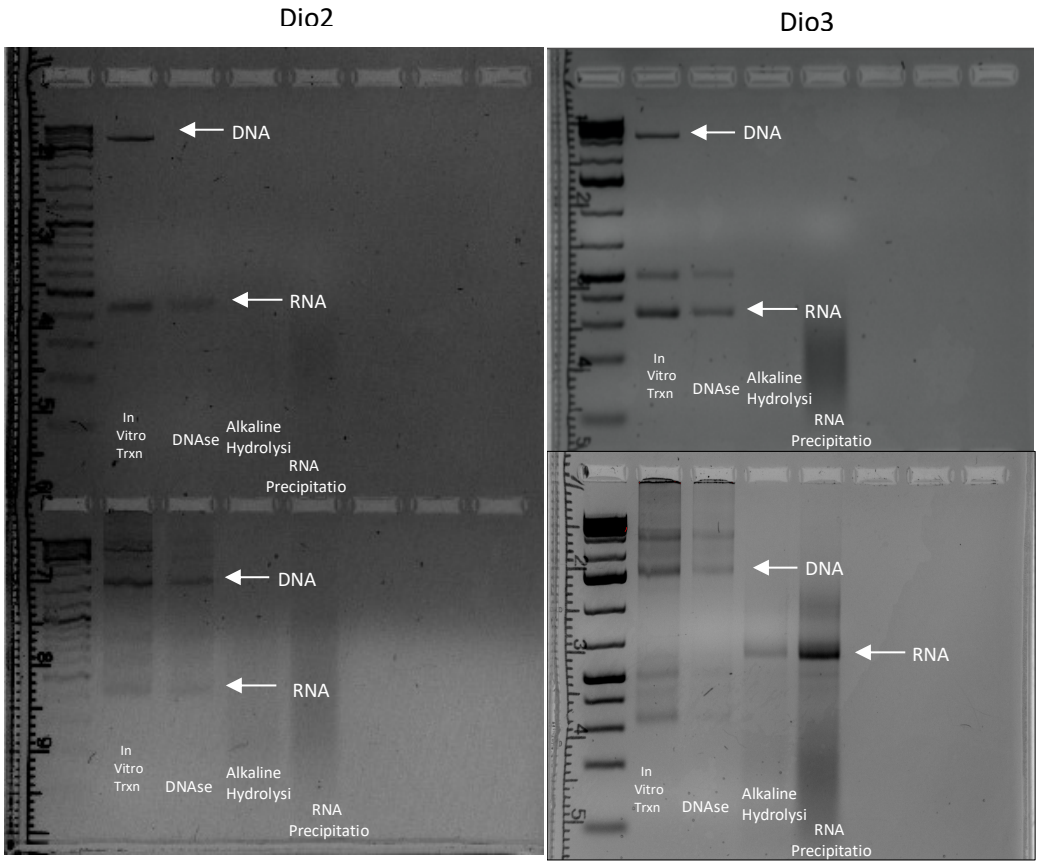

Figure 7. Processing of Dio2 and Dio3 sense and antisense probes. DNA was transcribed to yield RNA probes. RNA polymerases were used for reverse transcription (Trxn). For dio2, T3 was used for anti-sense and T7 for sense strand. For dio3, SP6 was used for anti-sense and T7 for sense strand. RNA was then treated with DNAseI, followed by alkaline hydrolysis and

DNA template. Sample of the probes prior to and after DNAse I addition were collected and electrophorosed on a gel to verify DNA removal.

Alkaline Hydrolysis and RNA Precipitation of RNA Probes

Na2CO3 and NaHCO3 were added to each reaction at a final concentration of 0.24M and incubated at 60°C for 20 minutes. A sample was saved of the hydrolysis for gel analysis. The probe RNA was precipitated with sodium acetate to a final concentration of 0.075 M. Pre-chilled 100% ethanol was added such that it is 2.5x the volume in each tube. Samples were placed at -80°C overnight. Samples were centrifuged at 13,000rpm for 30 minutes at 4°C, then washed with 70% ethanol, and resuspended in nuclease free water. Samples of each probe were collected for gel analysis. Concentrations of probes were determined spectroscopically using a nanodrop. Probes were diluted in hybridization buffer to a final concentration of 20ng/µL and stored at -80°C.

Hybridization

Prehybridization

Slides were removed from -80°C, thawed at room temperature, and washed 3x3 minutes in 1xPBS. Slides were acetylated using 0.1M TEA (triethanolamine)/0.25% acetic anhydride for 10 minutes followed by incubation in 0.1% Triton X-100 in 1xPBS for 30 minutes. Slides were washed 3x5 minutes in 1x PBS, then covered in hybridization buffer without probe and allowed to prehybridize for 2-4 hours in a humidity chamber with 50%

formamide/ 0.2xSSC soaked paper towels. All prehybridization washes and incubations were carried out at room temperature.

Hybridization.

In situ hybridization were performed with antisense and sense Dio3 and Dio2 probes. Dio2 and Dio3 probes were diluted to 1000 ng/mL in hybridization buffer and heated at 80°C for 5 minutes to remove secondary RNA structures, then placed on ice. Probe-hybridization buffer mixture was added to each slide, cover slipped and allowed to hybridize overnight at 68°C in a humidity chamber with 50% formamide/0.2X SSC (saline-sodium citrate) soaked paper towels.

Post Hybridization

Slides were washed in pre-warmed 0.2xSSC (changing solutions after 5 minutes, then 3x15 minutes) in a 68-72°C water bath. Slides were cooled and washed in room temperature 0.2xSSC for 5 minutes. Slides were then separated by probe for all SSC washes.

Antibody and Alkaline Phosphate Detection

Slides were washed with maleic acid buffer (MB) for 10 minutes, and then incubated in 2% MBMB (10% Boehringer Mannheim blocking buffer in MB) for 1 hour. Anti-digoxigenin-AP antibody (Roche) was diluted 1:2000 in 2%MBMB and added to each slide in a humidity chamber. Slides were incubated overnight at room temperature. The next day, slides were washed 2x30 minutes in MB, 2x30 minutes in color reaction buffer (Tris pH 9.5, 5M NaCl, 1M MgCl2, Tween 20 in H2O) and covered in NBT/BCIP (p-

nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate) (Roche) solution diluted 1:50 in color reaction buffer. Slides were incubated with color substrate for 18-24 hours in a tin foil-covered humidity chamber. Slides were washed in 1xPBS for 10 minutes and cover slipped with VectaMount AQ Aqueous Mounting Media (Vector Laboratories), allowed to dry at room temperature overnight, and then imaged on an Olympus BX53 brightfield microscope. All photos were taken of regions in middle cochlear turns unless otherwise stated.

Data Analysis

Two-way ANOVA was performed on RT-qPCR and ELISA to determine if there is an effect of age and treatment and whether age and treatment interact. A Sidak post hoc test was used to determine significant differences between individual comparisons. A two-tailed unpaired students t- test was also used to determine whether there were significant differences between sexes.

Results

Effect of treatment on body weight

Mice treated with thyroid gland inhibitors had significantly lower body weight at ages P5, P10 and P15 (Fig. 8A). A two-way ANOVA indicated an effect of age ($F_{(3,82)}$=343.7, p<0.0001****) and treatment ($F_{(1,82)}$=32.86, p<0.0001****) but no

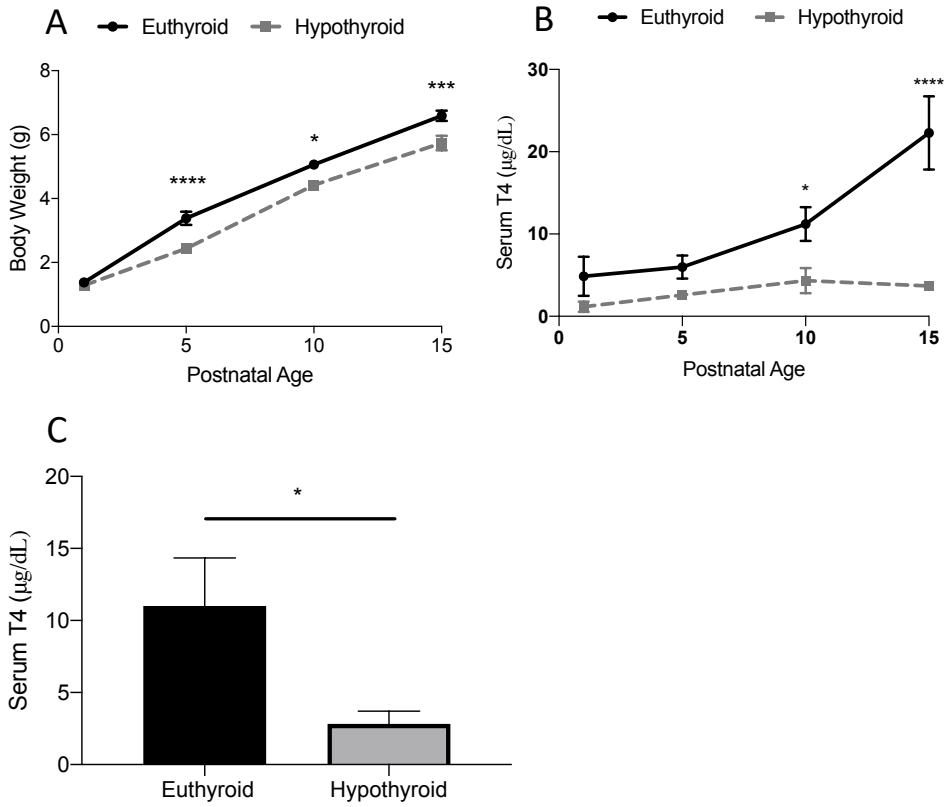

Figure 8. Effect of treatment on body weight. (A) Body weight of both male and female pups at different ages through cochlear development (n=24-28 per age per treatment). Hypothyroid pups showed significantly lower weight at age P5 (****p<0.0001), P10 (*p=0.0128), P15(***p=0.0006) compared to controls. **(B)** Serum T4 throughout development was measured by ELISA (Pooled P1 serum n=4, Pooled P5 serum n=9, Individual P10 and P15 serum n=11 each) Sidak's multiple comparisons demonstrated significantly lower total serum T4 in control versus hypothyroid pups at ages P10 (*p= 0.0475) and P15 (****p<0.0001). **(C)** Dam serum T4 was assessed by ELISA (n=5 control, n=6 hypothyroid). Dam serum was collected upon final pup sacrifice (P15 for all control and hypothyroid dam serum tested). Dams were sacrificed when their pups were age P15. Comparison of control and hypothyroid dams was done using an unpaired, two-tailed t test. Dams treated with thyroid gland inhibitors had significantly lower serum T4 compared to control dams (*p=0.0297).

interaction between the two. No differences in the body weight between female and male pups of the same age was found (data not shown).

Total serum T4 was measured by ELISA to verify the efficacy of treatment. ELISA indicated significantly lower serum T4 in hypothyroid P10 and P15 pups compared to control pups (Fig. 8B). Two-way ANOVA showed a significant effect on age ($F_{(3, 63)}$ = 8.653, ****p<0.0001), treatment ($F_{(1, 63)}$ = 26.21, ****p<0.0001), and the interaction between the two ($F_{(3, 63)}$ = 5.704, *p=0.0016). A significant reduction in T4 was not observed at P1 or P5. Dam serum was analyzed to verify maternal hypothyroidism, indicating subsequent hypothyroidism in offspring given that TH passes from maternal circulation to pups through placental transfer (Stepien and Huttner 2019). As expected, control dam serum had significantly higher T4 compared to hypothyroid dams (*p=0.0297) (Fig. 8C).

Quantification of Dio2 mRNA in euthyroid and hypothyroid cochlea

qRT-PCR demonstrated that the expression of Dio2 mRNA levels in a euthyroid cochlea peaked at P10, then decreased to almost the same level as P1, where it had its lowest expression (Fig. 9). In contrast, mRNA levels in the hypothyroid cochlea were greatest at

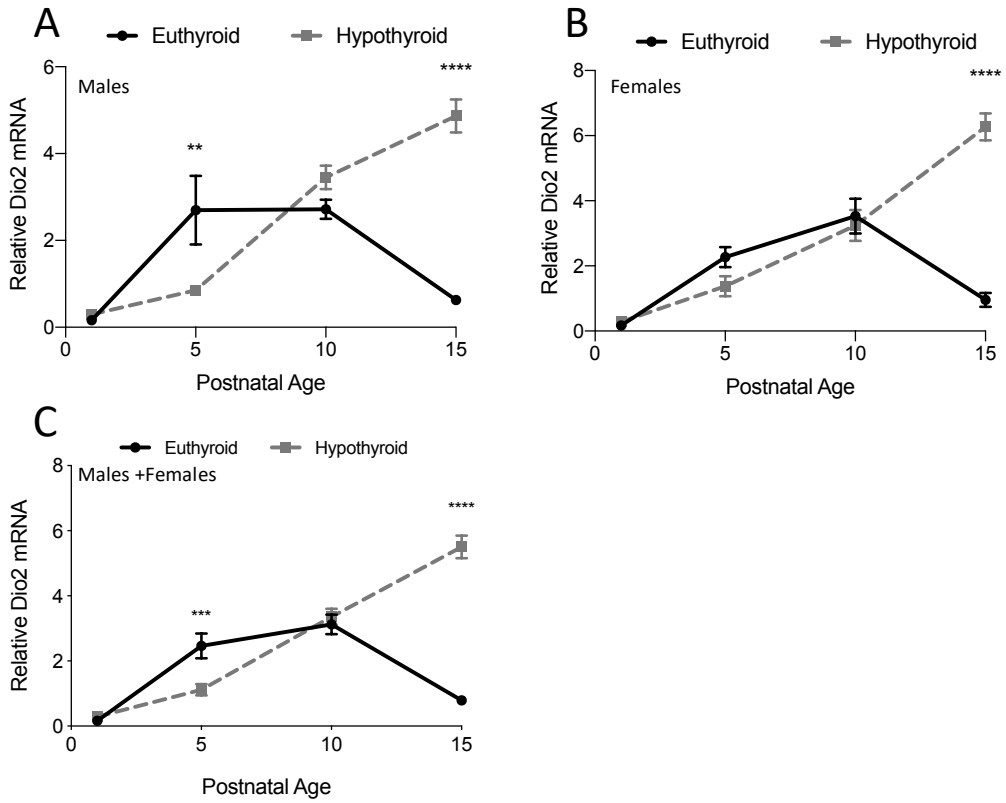

Figure 9. Dio2 mRNA expression during early postnatal development. (A) Male control individuals had highest expression at P5, with a decrease through P15 (n= 6 male, per age, per treatment). Hypothyroid male individuals had an increase of Dio2 expression at P10 to the highest level at P15.Comparison of both groups found significantly higher Dio2 mRNA expression in control pups at P5 (**p=0.0015) and higher expression in hypothyroid pups at P15 (****p<0.0001). **(B)** Female control individuals had highest expression at P10, with a decrease through P15 (n= 4-6 female, per age, per treatment). Hypothyroid female individuals had a steady increase of Dio2 expression starting at P5 to the highest level at P15. Comparison found significantly higher Dio2 mRNA expression in hypothyroid pups at P15(****p<0.0001). **(C)** Control individuals had highest expression level at P10, with a decrease through P15 (n=6 male and female, per age, per treatment). Hypothyroid individuals had an increase of Dio2 expression from P5, P10 to the highest level at P15.Comparison of both groups found significantly higher Dio2 mRNA expression in control pups at P5 (***p=0.0009) and higher expression in hypothyroid pups at P15 (****p<0.0001).

P15. Dio2 mRNA levels were lowest at P1 in the euthyroid and hypothyroid group. Two-way ANOVA test were performed individually for males and females: Males showed an effect on age ($F_{(3, 35)} = 24.30$, p<0.0001****) and treatment ($F_{(1, 35)} = 11.29$, p=0.0019**)

as well as a significant interaction between the two ($F_{(3, 35)} = 30.53$, $p<0.0001$****) ; Females showed an effect on age ($F_{(3, 35)} = 35.61$, $p<0.0001$****) and treatment ($F_{(1, 35)} = 17.55$, $p=0.0002$***) as well as a significant interaction between the two ($F_{(3, 35)} = 33.15$,$p<0.0001$****) (Fig. 9A,B). No differences were detected between male and female pups within pups of the same age and treatment group. However, for simplicity, male and female data was pooled since the data show the same trend whether sexes were pooled or separate. Two-way ANOVA test of pooled data indicated an effect on both age ($F_{(3,78)}=54.56$, $p<0.0001$****) and treatment ($F_{(1,78)}=26.16$, $p<0.0001$****), as well as a significant interaction between the two ($F_{(3,78)}=56.47$, $p<0.0001$****) (Fig. 9C). At P5, Dio2 expression was significantly lower in hypothyroid pups compared to the euthyroid controls for males and combined analysis (Fig. 9C) However, at P15, hypothyroid pups had a significantly higher expression of Dio2 than the euthyroid control. Dio2 mRNA levels between control and treated animals were observed at P1 or P10 (Fig. 9C).

Localization of Dio2 mRNA in the developing euthyroid and hypothyroid cochlea

In situ hybridization was performed to determine the spatial patterns of D2 mRNA at the window where the switch in expression of D2 takes place postnatally. D2 mRNA expression was present in the periosteal connective tissue in the modiolus, the

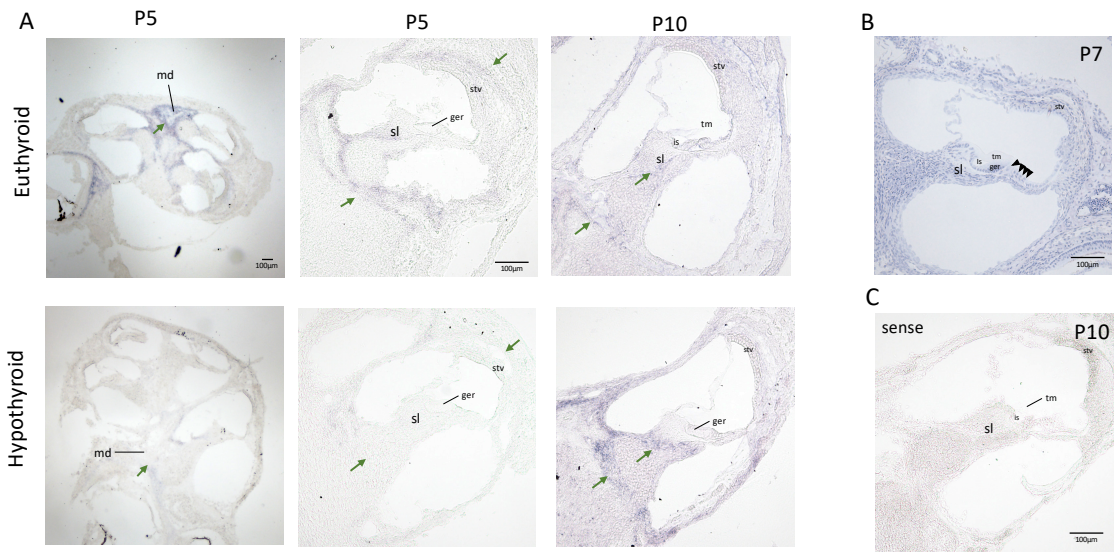

Figure 10. Dio2 mRNA expression in the developing cochlea. (A) Comparison of D2 mRNA presence in in the periosteal connective tissue in the modiolus, the lateral wall and the spiral lamina. D2 mRNA was expressed in a higher amount in the euthyroid cochlea then the hypothyroid at P5. The opposite was observed at P10. Both ages show delayed remodeling events including the prevalence of ger cells and small inner sulcus. (B) Hematoxylin stain of euthyroid cochlea at Postnatal day 7. Black arrow heads, outer hair cells (OHC). (C) P10 control D2 sense probe. Any staining in the sense is considered background. Abbreviations: md, modiolus; sl, spiral lamina; *ger*, greater epithelial ridge; *is*, inner sulcus; *tm, tectorial membrane; stv, stria vascularis.*

lateral wall and the spiral lamina. At postnatal day 5 Dio2 mRNA expression was visually higher in the euthyroid cochlear tissues compared to hypothyroid tissues; consistent with qRT-PCR results (Fig. 10 A). At postnatal day 10 Dio2 mRNA expression was higher in the hypothyroid cochlea compared euthyroid. Additionally, the hypothyroid cochlea shows a delay in the remodeling events of the cochlea as shown by the absence of a defined tectorial membrane, a smaller opening of the inner sulcus and the prevalence of GER cells (Fig 10A).

Quantification of Dio3 mRNA in euthyroid and hypothyroid cochlea

qRT-PCR demonstrated that the expression of Dio3 mRNA levels in the euthyroid cochlea were generally stable with a slight peak around P5. In the hypothyroid cochlea, levels were also stable with a dip at P5 (Fig.11). Two-way ANOVA test indicated no effect of age or a significant interaction for individually tested males and females. Additionally, no

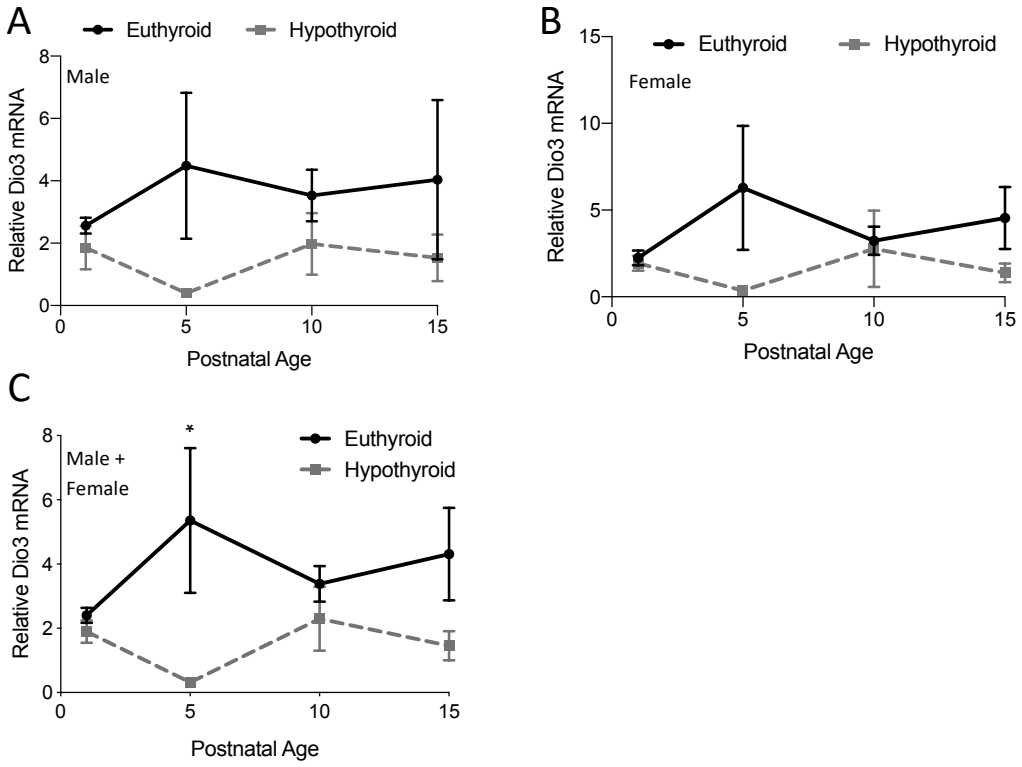

Figure 11. Dio3 mRNA expression during early postnatal development. (A) Control male individuals had highest expression level at P5, with a slight decrease at P10 followed by increasing at P15. (n=4-6**)** Hypothyroid male individuals had lower Dio3 expression at all ages with the highest level at P10. **(B)** Control female individuals had highest expression level at P5, with a slight decrease at P10 followed by increasing at P15. (n=3-6**)** Hypothyroid female individuals had lower Dio3 expression at all ages with the highest level at P10. **(C)** Control individuals had highest expression level at P5, with a decrease at P10 followed by increasing at P15. (n=6 male and female, per age, per treatment). Hypothyroid individuals had lower Dio3 expression at all ages with the highest level at P10. Comparison of both groups found significantly higher Dio3 mRNA expression in control pups at P5 (*p=0.0171).

differences were detected between male and female pups within pups of the same age and treatment group. For simplicity, male and female data was pooled since the data show the same general trend whether sexes were pooled or separate.

Two-way ANOVA test indicated no effect of age or a significant interaction for the combined analysis. However, there was an effect on treatment ($F_{(1,70)}=7.828$, $p<0.0066^{**}$) when both sexes were combined. Overall, Dio3 mRNA levels were lower in hypothyroid cochlea compared to euthyroid cochlea. *Post hoc* analysis reported at P5 that Dio2 expression was significantly lower in hypothyroid pups compared to the euthyroid controls (Fig. 11C).

Discussion

This study was conducted to investigate the expression of deiodinase enzymes in the developing cochlea when normal thyroid hormone levels are disrupted. Thyroid hormone must be available during critical developmental windows prior to the onset of auditory function because at later stages auditory deficits cannot be reversed by thyroid hormone supplementation (Ng et al. 2004). We aimed to evaluate whether deiodinase enzymes, D2 and D3, respond to perturbations in TH levels in a manner that is consistent with a compensatory mechanism capable restoring TH action in cochlear tissue. Such a response may protect the developing tissue from malformation under mild-moderate hypothyroid conditions. Our results are partially inconsistent with this idea and suggest that developmental programs that control deiodinase expression in development may be dominant over adaptive mechanisms that are thought to respond to perturbations in serum TH levels.

Compensation as a rescue mechanism

Considering the importance of thyroid hormone for normal development, there is the assumption that developing tissues hold potent compensatory mechanisms that serve to protect against small to moderate changes in circulation TH (Sharlin et al. 2010). This idea comes from the observation that T4 is secreted at a 10-times bigger volume than T3 (Graham et al. 2011), which indicates deiodinases must be present to regulate the tissue-level availability of active TH.

In fact, the deiodinases contribute to around 60% of all circulating T3 in rodents and 80% in humans suggesting that an interference to the expression of these enzymes will result in major problems with the serum T3 homeostasis (Abdalla and Bianco 2014). Therefore, considering that these enzymes are developmentally regulated, any alteration or delay in deiodinase expression may prevent these enzymes from not only functioning normally to promote tissue development, but may also prevent the deiodinases from working as adaptive mechanisms aimed at compensation.

Deiodinase II role in compensation

In the brain, D2 is proposed to function as a protective mechanism against hypothyroidism due to observed increases in enzymatic activity and mRNA levels in response to low T4 conditions (Giadano-Ferraz et al. 1999). Particularly in neural systems, this protective system seems to be operating more efficiently due to their critical need for TH. Similarly, it has been reported that the post-natal cochlea has important T3 demands given that specific cochlear developmental events such as the formation of the tectorial membrane, opening of the inner sulcus, and maturation of sensory epithelium require temporal TH action (Deol 1973; Rusch et al. 2001; Ng et al. 2004). Therefore, D2 may serve to protect these TH-dependent events of the cochlea against fluctuations in TH levels that occur in hypothyroidism.

Ng et al. (2004) observed cochlear malformations upon deletion of D2 gene. Inactivation of D2 resulted in delayed differentiation of known TH sensitive developmental events including opening of the inner sulcus and malformation of the tectorial membrane.

These malformations were also observed in our in-situ hybridization (Fig. 10). Interestingly, these defects occur in tissues that not express D2 (Campos-Barros et al. 2002). This observation suggests that proper expression of D2 in these surrounding tissues is necessary for proper cochlear development and auditory function (Ng et al. 2004).

Our quantification studies demonstrate that the post-natal surge in dio2 mRNA expression is delayed in the hypothyroid cochlea. Importantly, at post-natal day 5 the level of mRNA expression of dio2 is significantly lower than the control (Fig. 9C). This suggests that even if sufficient T4 was reaching the tissue to prevent malformation, the system is unable to utilize it due to its inability to activate it. Therefore, in the mouse cochlea, D2 cannot act as a compensatory mechanism until post-natal day P10 where dio2 mRNA levels surpass the control. At this point, the tissue malformation will persist because even with super physiological doses of T3 there are no significant changes to the morphology of the tissue (Ng et al. 2004). Sharlin et al (2010), reported dio2 mRNA levels in the brain were elevated in the hippocampus of P14 pups with 82 % reduction in total T4 serum. Our findings show dio2 mRNA expression was significantly higher than the control the cochlea at post-natal day 15 (Fig.9). This observation suggests that as development proceeds, D2 mRNA expression may eventually be sufficient to respond in a manner consistent with compensation.

Deiodinase III role in compensation

Type 3 deiodinase enzyme functions to deplete active sources of thyroid hormone. With that, it has been proposed to play a protective role in auditory development by

preventing premature TH action in the developing cochlea. Studies in D3 knockout mice showed that D3 activity is imperative for normal development of the cochlea. In the absence of D3, an acceleration of cochlear development was observed that could be phenocopied by providing provisions of excess T3. This observation indicated that in the absence of D3, the cochlea is prematurely exposure to T3 which ultimately will lead to malformation of cochlea and deafness (Ng et al. 2009).

We hypothesized that normal early postnatal decrease in D3 mRNA expression would be delayed. Contrary to our hypothesis, we observed that hypothyroidism induced a general reduction of D3 mRNA levels in the developing cochlea. Particularly, at postnatal day 5, D3 mRNA levels were found to be significantly reduced in the hypothyroid cochlea compared to the euthyroid cochlea. This was also the age where we observed a peak in D3 mRNA in the control group (Fig. 11). Interestingly, Ng et al. (2009) reported D3 levels to be low postnatally with no transient postnatal peak similar to the present study. Nonetheless, this report argued that the elevated Dio3 prenatally restricts TH action by reducing TH availability. Although it is not immediately why our study observed postnatal peak in Dio3 mRNA and the previous study did not, it might be due to differences in assay sensitivity. The present study utilized highly sensitive qRT-PCR where the previous study utilized northern blot. Additionally, the experimental ages between studies do not perfectly match-up. Therefore, our observed bump in expression at P5 may have been missed previously. Nonetheless, the persistent reduction in Dio3 mRNA levels observed in the hypothyroid cochlea is indicative of an adaptive response that could be attempting to compensate for the goitrogen-induced reductions in T4. Taken together, it appears as

though Dio2 and Dio3 regulation during cochlear development as well as their ability to respond in a predicted manner according to the compensation hypothesis are differentially regulated.

Future studies

From the above information it can be concluded that development of the cochlea and normal auditory function requires a tightly controlled and temporally regulated availability of T3. This is achieved by synchronized and developmentally inverted expression of D2 and D3 (Williams and Bassett 2011; Ng et al. 2013; Sharlin et al. 2010). However, it is still unknown what is mediating this switch in expression of D2 and D3 during development of the cochlea.

Histone modifications

A plausible next step is investigating whether modifications to histones, such as methylation and acetylation, are the underlying mechanism to the switch in D2 and D3 expression. Ambrosio et al reported in 2013 that LSD-1, a lysine-specific histone demethylase, is essential for transcriptional induction of D2 and repression of D3 during myogenesis. Interestingly, they reported that demethylation of H3-K9 on the Dio2 promoter might be the molecular mechanism underlying an increase in D2 expression that is observed during the start of myogenesis (Ambrosio et al. 2013). It would be interesting to investigate whether this holds true for the deiodinase expression in the developing cochlea. We hypothesize that in hypothyroid mice, methylation of H3-K9 on the D2

promoter will remain high longer than euthyroid mice, mirroring the delay in D2 mRNA observed in our studies, preventing the proper remodeling of auditory structures, and ultimately hearing dysfunction. Changes in activating histone acetylation marks would be expected to accompany the changes in methylation.

With regard to D3, an opposite pattern to that of D2 would be expected with low D3 promoter histone methylation during the prenatal period and higher levels of methylation postnatally. Considering that D3 mRNA levels responded to goitrogen treatment in manner that would be expected for compensatory mechanism (in contrast to D2), it would be interesting to determine whether histone modifications are altered in a developmentally hypothyroid state.

Enzyme activity

Measuring mRNA expression is not the same as measuring enzymatic activity. Previous studies have demonstrated that in the developing euthyroid cochlea mRNA expression tends to peak before the enzyme activity (Ng et al. 2009). Additionally, Sharlin et al (2010) found that D2 activity in the brain was also elevated as a result low TH conditions and concluded that D2 cannot fully protect the developing brain from a reduction in TH action (Sharlin et al. 2010). Therefore, it will be important to measure enzyme activity and determine how cochlear enzymatic activity responds to a low TH state. It would not be surprising if activity changes were observed; supporting the idea that D2 has a role in acting as a compensatory mechanism in development.

Interestingly, the D2 and D3 pathway, and thus the tissue-specific TH signaling is regulated by ubiquination (Sagar et al. 2007; Bianco and Conceição, 2018). Ubiquination

is a critical, post-translational regulator of protein stability. It is also able to regulate enzyme activity by transiently inactivating enzyme function through conformational changes which can be reversed upon deubiquination (De Castro et al. 2015; Sagar et al. 2007; Gereben et al. 2000). In the chicken developing growth plate, Hedgehog signaling inhibits D2 mediated T3 production by inducing WSB-1, a ubiquitin ligase that targets D2 dimers. Interestingly, at the same time, hedgehog signaling stimulates D3 expression. (Dentice et al. 2005; Sagar et al. 2007). Sagar et al (2007) reported that ubiquination is able to inactivate D2 enzyme by transiently interfering with its dimeric conformation. Additionally, they reported the ability of VDU-1 based catalytic core complexes to trigger the removal of this initial ubiquination to favor a model where dimeric D2 is de-ubiquinated rather than being assembled every time (Sagar et al. 2007). This suggested model represents a physiological advantage that might provide a moderate compensation effects in developing tissues such as the cochlea. Under the assumption that ubiquinated enzymes might be able to respond faster to changes in TH availability than mRNA because of the de-ubiquination reaction requires less than transcription and translation.

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