Cochlear Development And Auditory Function In The Absence Of Thyroid Hormone Transporters Mct8 And Oatp1c1

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Cochlear Development And Auditory Function In The Absence Of

Thyroid Hormone Transporters Mct8 And Oatp1c1

By

Richard J. Sinn

A Thesis Submitted in Partial Fulfillment of the

Requirements for the Degree of

Master of Science

In

Biology

Minnesota State University, Mankato

Mankato, Minnesota

November 2014
Cochlear Development and Auditory Function In The Absence of Thyroid Hormone Transporters Mct8 and Oatp1c1

Richard J. Sinn

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

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Dr. David Sharlin (Advisor)

__________________________________  
Dr. Geoffrey Goellner

__________________________________  
Dr. Michael Bentley
Abstract

Cochlear Development and Auditory Function in the Absence Of Thyroid Hormone Transporters Mct8 and Oatp1c1

Name: Richard John Sinn
Degree: Master of Science in Biology
Institution: Minnesota State University, Mankato
Mankato, Minnesota, 2014.

Thyroid hormone (TH) is essential for the development and maturation of the nervous system. The thyroid gland secretes an active form of TH, triiodothyronine, and a prohormone, thyroxine, into the blood. TH is charged, which prevents it from passively diffusing across cell membranes and thus requires cell membrane transporters to facilitate its movement into and out of cells. However, whether TH transporters are required for TH-mediated developmental events, including the auditory system, is largely unknown.

The purpose of the present study was to investigate two specific TH transporters, monocarboxylate transporter 8 (Mct8) and organic anion transporting polypeptide 1c1 (Oatp1c1), and their role in the development and function of the auditory system. Development was observed by comparing key structures within the cochlea necessary for normal hearing between mice lacking each transporter individually or in combination by taking measurements from histological sections. Histological analysis demonstrated no significant difference in the size of the tectorial membrane, tunnel of Corti, or greater epithelial ridge between any of the genotypes. To test the auditory function and the integrity of the auditory pathway, auditory-evoked brainstem response (ABR) was evaluated. ABR data demonstrated that mice lacking both Mct8 and Oatp1c1 (double KO
mice), as compared to wildtype, had significantly elevated hearing thresholds at 32kHz, but not at 8kHz or 16kHz. Furthermore, analysis of ABR waveforms demonstrated significantly slower latencies at all frequencies tested. Taken together, these results suggest cochlear structure is largely normal in the absence of TH transporters MCT8 and Oatp1c1, but that these transporters play an essential role in the processing of auditory signals through the brain stem.
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I would also like to thank Dr. Michael Bentley and Dr. Geoffery Goellner for being excellent committee members. They have given me a wealth of knowledge that has been of great benefit both academically and professionally.

Last, but not least, I would like to thank my fellow graduate students who have been there to help me through the good times and the bad times. I would not have been able to make it through graduate school without them.
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<tr>
<td>T4</td>
<td>Thyroxine</td>
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<tr>
<td>T3</td>
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<td>TH</td>
<td>Thyroid Hormone</td>
</tr>
<tr>
<td>TR</td>
<td>Thyroid Hormone Receptors</td>
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<tr>
<td>Mct8</td>
<td>Monocarboxylate Transporter 8</td>
</tr>
<tr>
<td>Oatp1c1</td>
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<td>Decibel</td>
</tr>
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<tr>
<td>Spl</td>
<td>Sound Pressure Level</td>
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<td>Allan-Herndon-Dudley Syndrome</td>
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<tr>
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<td>Proteinase K</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris/Acetate/EDTA Buffer</td>
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<tr>
<td>GER</td>
<td>Greater Epithelial Ridge</td>
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Introduction

Thyroid hormone (TH) is commonly considered essential for regulating metabolism, but what is less appreciated is its role in-coordinating development. Children born to mothers with low TH levels or with an underactive thyroid gland face a number of neurological impairments ranging from mental retardation to deafness. These deficits likely result from a disturbance in a developmental program controlled by TH action.

TH action in development is regulated at multiple levels including the presence or absence of thyroid hormone receptors, deiodinases enzymes that activate or inactivate TH, and transporters that facilitate the uptake and efflux of thyroid hormone across cell membranes. Previous studies have shown the importance of thyroid hormone receptors (TRs) and deiodinase enzymes in development of the cochlea and hearing function (2-4). Specifically, in mice lacking TRs, cochlear development is delayed and auditory function is disturbed. Similarly, in mice lacking type 2 deiodinases, an activating enzyme that converts the prohormone thyroxine (T4) to the major receptor ligand triiodothyronine (T3), cochlear development is delayed and auditory function is disturbed. Together these studies demonstrate that a local amplification of thyroid hormone signaling is required for normal cochlear development.

Membrane transporters are required for cellular uptake, and efflux, before thyroid hormone can be activated by type 2 deiodinases or bind to receptors. There are multiple thyroid hormone transporters in the cochlea, but the two that we are interested in are monocarboxylate transporter 8 (Mct8) and organic ion transporting polypeptide 1c1.
(Oatp1c1) as they have a complimentary expression pattern. Preliminary studies suggest that cochlear development and auditory function are normal in mice lacking either Mct8 or Oatp1c1. However, considering that both Mct8 and Oatp1c1 mediate T4 transport and in situ hybridization has shown an overlap in their expression within the cochlea, functional compensation between these two transporters may exist (5). Therefore, the working hypothesis is that: mice lacking both thyroid hormone transporters, Mct8 and Oatp1c1, will have significantly higher hearing thresholds and delayed cochlear development, consistent with hypothyroidism, as compared to wildtype mice.

Literature Review

A. Control of thyroid hormone action

A.1 – Thyroid Hormone (T3 and T4)

The thyroid is a butterfly-shaped gland located on the anterior surface of the trachea and is responsible for the synthesis and secretion of two hormones: tetraiodothyronine, or thyroxine, (T4) and 3, 5, 3’-triiodothyronine (T3) (6).

TH is synthesized in the thyroid by adding three or four iodine atoms to pre-existing tyrosine amino acids on thyroglobulin proteins. T4 is considered a prohormone because it binds the thyroid hormone receptor with little affinity, but is the dominant hormone released by the thyroid gland and is converted locally within tissues via deiodinases into T3, the active form, which binds to TRs activating or repressing gene expression in cells (7).
TH concentrations in the blood are regulated by the hypothalamus, pituitary gland, and the thyroid gland, or by what is more commonly called the hypothalamic-pituitary-thyroid axis (HPT-axis). The hypothalamus releases thyrotropin releasing hormone (TRH) which stimulates the pituitary gland to release thyroid stimulating hormone (TSH) which stimulates the thyroid gland to synthesize and secrete more T4 and T3. If TH concentrations in the blood get too high or too low the HPT-axis can use a classic system of negative feedback loops to regulate TH levels back to normal (6). If excess TH is circulating in the blood, then TH signals to the hypothalamus and pituitary gland to inhibit the synthesis and secretion of TRH and TSH, respectively, thus inhibiting the secretion of more TH into the blood. Conversely if there is too little TH in the blood, the body will try to counteract this condition by secreting more T3 and T4, by increasing TRH or TSH secretion (6).

A.2 – Receptors

The majority of the biological actions controlled by TH are mediated by TRs. TRs are located in the nucleus and are ligand dependent transcription factors that control the growth and differentiation of central nervous system (CNS) cells (8). There are two TR genes: TRα and TRβ. While each type of receptor has its own function and precise tissue expression, they both share a high affinity for T3. TRα1 is the major receptor expressed in the brain during fetal development and has shown to play a secondary role in development of the cochlea (9). TRβ is processed into two isoforms: TRβ1 and TRβ2. TRβ1 is expressed widely throughout the body, including the cochlea, and is considered
the major TR responsible for TH-mediated cochlear development (4). TRβ2 expression is more restricted with expression reported only in the brain, pituitary, and cochlea (10).

A.3 – *Deiodinase Enzymes*

The function of an iodothyronine deiodinase enzyme is to remove an iodine atom from iodothyronines, thus activating or inactivating TH (11). For the purpose of this study, its main function is to either remove an iodine atom from T4 to make an active T3 ligand or to inactivate T4 into reverse T3 (rT3). There are three main deiodinases that interact with TH: Type 1 deiodinase (D1), Type 2 deiodinase (D2), and Type 3 deiodinase (D3) (11). D1 removes an iodine atom from the inner ring of TH to make a T3, rT3, or T2 depending on the substrate available; this process mainly occurs in the liver and kidney (12). D2 removes the 5’ iodine atom from the outer ring of T4 to create T3 (Figure 1) (13). D3 removes the 5’ iodine from the inner ring changing T4 into rT3 or by changing T3 into T2 (Figure 1) (11).
A.4 – Transporters (Mct8, Oatp1c1)

THs do not passively diffuse across the membrane due to their highly charged amino acid side chain. Thus, TH requires transporters to regulate the diffusion of TH from serum or cerebral spinal fluid across plasma membranes into or out of cells (14). TH transporters, such as monocarboxylate transporter 8 (Mct8), allow for facilitated diffusion of both T3 and T4, or they can be specific and transport only a single iodothyronine like organic ion transporting polypeptide1c1 (Oatp1c1) that is T4 selective (15,16). Mct8 is expressed in multiple tissues but, in particular, areas that are sensitive to TH such as the developing brain and cochlea (5). Oatp1c1, like Mct8, also localizes to the cochlea (5).

Regulation of the movement of T3 and T4 becomes important when discussing
development of sensory tissues or the CNS (5). It is essential that developing tissues see T4 or T3 at specific times otherwise they will experience abnormal growth due to local hypothyroid or hyperthyroid conditions (17). Human mutations in the Mct8 gene result in elevated serum T3, below normal serum T4, severe mental retardation, and other neurological impairments (17,18). These observations strongly suggest that Mct8 transporters are important in development.

B. Auditory System

B1.1 – Auditory functions

The auditory pathway starts when sound waves hit the tympanic membrane of the middle ear. Not all sound waves are created equally; each has its own amplitude, which is measured in decibels (dB) and pitch or frequency (Hz). The average human can hear in the range of 20 to 20,000 Hz (7). The process of hearing takes place in the inner ear which is comprised of four main parts: the cochlea, semicircular canals, vestibular apparatus, and the vestibular cochlear nerve (VIII) (19). When sound waves hit the tympanic membrane it will start to vibrate causing the ossicles on the other side to move and create vibrations through the oval window and into the fluid-filled cochlea. This is important because the sound waves have now changed medium from air to fluid where they can be detected (6).

The cochlea is a spiral shaped fluid-filled sound receptor. It has the ability to change sound waves from the environment into electrical impulses that the brain can interpret. It is comprised of three fluid-filled chambers that spiral around the cochlea and
end at the apical surface (19). Two outer fluid-filled chambers that surround the inner chamber are the scala vestibule and scala tympani; both are filled with a sodium-ion-rich fluid called perilymph (19). The inner fluid-filled chamber is called the scala media and it contains a more potassium-rich fluid called endolymph (19). What distinguishes the scala media from the other two compartments is that it contains the Organ of Corti, which consists of a basilar membrane and hair cells. There are approximately 16,000 total hair cells arranged in four rows divided in to a single row of inner hair cells and three rows of outer hair cells (7). The inner hairs are the primary cells responsible for hearing while the outer hair cells amplify quieter sounds (7). The very tips of the hair cells are called stereocilia and they are embedded in the tectorial membrane, which, when it moves, will cause the stereocilia and hair cells to bend in one direction or another (6). Hair cells act like mechanically gated channels so when stereocilia move in one direction they open, allowing the potassium in the endolymph to rush into the hair cells causing them to depolarize (6). When hair cells depolarize sequestered calcium ions are released creating action potentials that stimulate the Vestibulocochlear nerve (VIII) (7). Louder sounds cause greater movements of the tectorial membrane. The more the stereocilia move the greater the depolarization, which allows for more calcium ions to be released and more action potentials to take place to stimulate the Vestibulocochlear nerve (6,19). The action potentials travel down neurons from the cochlea to the brainstem where they will synapse with second-order neurons that run to a nucleus of the thalamus. Here they synapse with a third-order neuron that will take the information to auditory cortex found in the temporal lobe of the brain (Figure 2) (6).
B1.2 – *Auditory Brainstem Response (ABR)*

Auditory-evoked brainstem response (ABR) is a functional analysis of hearing (20). ABR works by detecting voltage changes throughout the brainstem that have been evoked by sound. The presence of voltage changes, or lack of voltage changes, provides an indication of the functionality of the cochlea and auditory pathway. The output from ABR is the detection of five distinct voltage waves (Figure 3) with each wave related to a different neuroanatomical structure or nucleus in the auditory pathway (Figure 2). The waves are recorded in positive and negative voltages that are recorded over hundreds or thousands of ‘sweeps’ and eventually displayed as averages. The averages are used to help reduce any random background “noise” (20). The appearance or disappearance of these waves at varying sound pressure levels (SPL) can be used to measure the functionality of the individual’s hearing ability (20,21) (Figure 4).

The basic components are a computer, signal recorder, trig box, headphones, and electrodes. The computer is used to setup the SPL and frequency you want the stimulator
and headphones to produce. The signal recorder is connected to the electrodes to detect electrical changes and the computer to display the data that has been recorded. The trig box tells the signal recorder and the stimuli producer when to record data and produce sound, respectively (22). To measure the differences in the auditory pathway of mice, three electrodes must be placed. One electrode is subdermally inserted into the thigh as a ground, one electrode is placed in the scalp between the ears (active), and the third electrode (reference) is placed just behind the ear (Figure 3) (21).

Sound is channeled into the ears at varying frequencies and Spl to test auditory function. Frequencies can also be used to test functionality of different portions of the cochlea as high frequencies are heard in the basal portion of the cochlea while low frequencies are heard in the apical surface (6).

Figure 3. Standard ABR setup with electrodes subdermally attached to the mouse. The blue electrode is positioned at the vertex of the skull, the yellow is positioned at the mastoid below the ear, and the black electrode is positioned along the thigh. Sound is channeled into the ears of the mouse with high-frequency transducers.
B.2 – Thyroid hormone and auditory function

B2.1 – *Role of thyroid hormone in auditory development*

As discussed earlier, low levels of TH during development is known as hypothyroidism, which results in neurological dysfunction including low IQ, impaired motor skills, and malformations of their cochlea leading to impaired hearing (23,24). These deficits are typically seen in neonatal infants exposed to hypothyroid conditions during critical developmental periods. T3 is essential for the maturation and development of tissues, so if T3 levels are too low the development of tissues is delayed or doesn’t
occur properly (25). Experiencing hypothyroid conditions during key stages of development will delay development of the cochlea and lead to hearing loss (24,26).

How does lack of TH affect the structure of the cochlea? It has been shown, in mice, that being hypothyroid can cause the tectoral membrane to become distorted during development to the point that it doesn’t come into contact with the inner or outer hair cells (24). Thyroid hormone is responsible for cochlear maturation and development, so if a case of hypothyroidism is not treated early it can also lead to permanent auditory deficits (24).

B2.2 – *Role of thyroid hormone receptors in auditory development*

TRs are coded from either the ThrA or the ThrB gene, coding for either the alpha- or beta receptors respectively. Mutations to the ThrB gene are associated with deafness in mice (4), and cognitive dysfunction and hearing loss in humans (27). It has been shown that ThrB mutations can lead to retarded maturation of the hair cells found in the cochlea, which may help explain the loss of hearing (28). ThrA genes have not been studied to the extent of ThrB, but ThrA genes do not appear to be necessary for developing auditory function (28). Comparatively, when ThrB genes are knocked out, mice display an auditory dysfunction, have delayed cochlear remodeling, and a deformed tectorial membrane but not to the same extent as a developmentally hypothyroid animal (9,29). Mice with mutations to both ThrA and ThrB have been shown to display a phenotype that is consistent with a mouse that is hypothyroid by having a grossly enlarged tectorial membranes and impaired hearing (9). If the tectoral membrane is deformed it prevents it
from coming into contact with the hair cells and allowing sound vibrations from becoming electrical signals in the auditory pathway (9).

B2.3 – Role of deiodinase enzymes in auditory development

Deiodinases play critical roles in regulating the amount of T3 tissues are exposed to, thus preventing local hypothyroid or hyperthyroid conditions and abnormal cochlear development (30). If Dio2, the gene that codes for D2, is knocked out mice will have lower T3 levels in tissues (31). Although the levels of T3 and T4 in those mice were normal in serum, they were not able to use D2 to convert T4 into T3 locally in tissue, which results in delayed cochlear development and defective auditory function (2). This suggests D2 does not influence TH levels in the serum in mice, but is needed locally at the tissue level to convert T4 to T3. D2 activity peaks during postnatal day 7 and stays relatively steady until it sharply declines at postnatal day 10; if D2 does not get expressed, the tectoral membrane will not develop properly and its function in the helping bend the stereocilia of the organ of Corti will be compromised (2,9).

Dio3 is the gene that codes for D3 which is the enzyme that is responsible for inactivating thyroid hormone at the cellular level (3). Mice lacking Dio3 have accelerated cochlear development and severe auditory deficits likely due to an excess of tissue T3 suggesting Dio3 serves a protective role (3).
B2.4 – *Role of thyroid hormone transporters in auditory development*

Mct8 is one of the most widely-studied TH transporters. Mct8 is a sodium-independent and energy-independent transporter. Mice and human mutations in the MCT8 gene result in Allan-Herndon-Dudley syndrome (AHDS); a syndrome characterized by mental retardation, poor communication skills, and impaired motor function (18,32). It is hypothesized that these effects result from a reduced ability to transport T3 into or out of the cell. Within the cochlea, Mct8 mRNA has been shown to be expressed in the cells of the tympanic border, greater epithelial ridge (GER), and fibrocytes in the spiral limbus and spiral ligament; suggesting it may play a role in cochlear development (5). However, different studies report the presence or absence of hearing deficits in patients with MCT8 mutations (17,33). Unpublished preliminary studies indicate that mice lacking Mct8 have normal hearing function.

Another transporter of interest is Oatp1c1 (also known as Oatp14) as it plays a different role in development as a transporter of T4 across the blood brain barrier (34). Oatp1c1, like Mct8, is also a sodium-independent and energy-independent thyroid hormone transporter. Within the cochlea, Oatp1c1 mRNA has been detected in the tympanic border as well as fibrocytes in the spiral limbus and spiral ligament (5). It should also be mentioned that mice deficient in Oatp1c1 transporters have normal serum levels of T3 and T4, but decreased brain tissue T3 and T4 (34). Unpublished preliminary studies indicate that mice lacking Oatp1c1 also have normal hearing function.


Materials and Methods

**Mouse Strains.** The mice are inbred strain C57BL/6J provided by collaborators from the Heuer lab in Germany. Desired genotypes (Table 1) were generated by breeding heterozygous female mice (Mct8<sup>+/−</sup>;Oatp1c1<sup>+/−</sup>) with male mice of the following genotypes: wildtype (Mct8<sup>+/+</sup>;Oatp1c1<sup>+/+</sup>), Oatp1c1 KO (Mct8<sup>+/+</sup>;Oatp1c1<sup>−/−</sup>), and Mct8 KO (Mct8<sup>−/−</sup>;Oatp1c1<sup>+/−</sup>).

<table>
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<th>Genotypes</th>
<th>Oatp1C1 Wildtype</th>
<th>Oatp1C1 Knockout</th>
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<td>Mct8 Wildtype</td>
<td>(Mct8&lt;sup&gt;+/+&lt;/sup&gt;;Oatp1c1&lt;sup&gt;+/+&lt;/sup&gt;)</td>
<td>(Mct8&lt;sup&gt;+/+&lt;/sup&gt;;Oatp1c1&lt;sup&gt;−/−&lt;/sup&gt;)</td>
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<td>(Mct8&lt;sup&gt;−/−&lt;/sup&gt;;Oatp1c1&lt;sup&gt;−/−&lt;/sup&gt;)</td>
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Table 1. Desired genotypes to be generated and investigated for cochlear development and auditory function using ABR and histology.

**Genotyping Using Polymerase Chain Reaction (PCR).** At one month of age, a 5mm tail clip was placed in a labeled Eppendorf tube and digested in 500µl of tail lysis buffer (TLB) and 5µl of fresh Proteinase K (PK) (stock solution of 200µg/ml). TLB consisted of 50mM Tris-Cl (pH 8.0), 20mM NaCl, 1mM EDTA, and 0.2% SDS. PK consisted of 10mM Tris-Cl (pH8.0), 20mM CaCl<sub>2</sub>, 50% Glycerol, and enough double deionized water (ddH<sub>2</sub>O) to bring everything to their desired concentrations. Tubes containing the mouse tails, TLB, and Proteinase K were placed in a thermal shaker at 56°C until the tails were fully digested. After digestion, tails were centrifuged at 14,000RPM to pellet hair and debris, 450µl of supernatant was removed and placed in a clean Eppendorf tube along with 450µl of 100% isopropanol to precipitate out the DNA. Precipitated DNA was dissolved in 200µl of ddH<sub>2</sub>O.
A PCR master mix for the Mct8 gene was made for each tail and control DNA, that consisted of 10µM of 0.5µl (final concentration 0.23µM) of primers Mct8-A, Mct8-B, and Mct8-C, 10µl of GoTaq® Green 2X Master Mix (final concentration 1X), and 8.5µl of ddH₂O (Table 2). A master mix for the Oatp1c1 gene was made, for each tail and control DNA, which consisted of 10µM of 0.5µl (final concentration 0.23µM) of primers Del For, Del Wt2, and Del Rev, 10µl of GoTaq® Green 2X Master Mix (final concentration 1X), and 8.5µl of ddH₂O (Table 2). 20µl of each master mix were pipetted into its own set of PCR tubes (one for Mct8 and one for Oatp1c1) and 1µl of DNA was added to each. PCR tubes were placed in a thermal cycler and cycled through a program that runs through seven steps. The steps were as follows: Step 1 - 95°C for 3 minutes, step 2 - 95°C of 30 seconds, step 3 - 58°C for 1 minute, step 4 - 72°C for 1 minute, step 5 – repeat steps 2 to 4 thirty-five times, step 6 - 72°C for 10 minutes, and step 7 – hold at 4°C. Figure 5 shows function of each primer plays in replicating DNA during PCR.

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<th>Primer</th>
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<td></td>
<td>Wt2</td>
<td>5’-CATCGCTTTGATGAGTGGTCTTG-3’</td>
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<td></td>
<td>C</td>
<td>5’-GGGCCAGCTCATTCTCCTCCCACTCAT-3’</td>
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</tbody>
</table>

Table 2. Primers used during PCR to amplify Oatp1c1 and Mct8 genes.
A 2% agarose gel was made with 2.4 grams of LE Agarose gel powder and 120ml of Tris/Acetate/EDTA buffer (TAE), heated in a microwave for 2-3 minutes, and swirled once to facilitate the mixing process. After the gel was mixed, 6µl of ethidium bromide was added (final concentration 0.5µg/ml). The gel mixture was allowed to cool for a few minutes before being placed in a mold to harden for 30 minutes to an hour. The finished gel was placed in a gel tank filled with 1X TAE and 10µl of ladder or 10µl of DNA were added to the wells, and run for 30-45 minutes at 80V. Once the gel was been completed it was be illuminated with UV light in a Kodak Gel Doc to

**Gel Electrophoresis.**
visualize the transporter banding. For Mct8, wildtype banding was seen at 400bp, knockout banding at 650bp, and heterozygotes at both 400bp and 650bp (Figure 6A). For Oatp1c1, wildtype banding was seen at 700bp, knockout at 550bp, and heterozygotes at both 700bp and 550bp (Figure 6B).

![Figure 6. Ethidium bromide stained agarose gels demonstrating Mct8 (A) and Oatp1c1 (B) and amplicons. For both figures, from left to right a knockout animal (-/-), heterozygous animal (+/-), wildtype animal (+/+), and a DNA base pair ladder (L).](image)

**Auditory-evoked Brainstem Response (ABR).** ABR testing was performed when the mouse was between 2-3 months of age to test the hearing threshold of each genotype of interest ((Mct8+/+, Oatp1c1+/+), (Mct8+/+, Oatp1c1+/−), (Mct8+/−, Oatp1c1+/+), (Mct8+/−, Oatp1c1+/−)) (Table 1). Each mouse was anesthetized with 1.2% Avertin injected intraperitoneally. The amount of Avertin given was based on weight. Hearing was tested with a series of pure tone stimuli at 8, 16, and 32 KHz at decreasing Spl to determine their frequency specific hearing thresholds.

**Dissection.** Considering that TH is important during development and cochlear development largely occurs after birth, removal of the cochlea was done at postnatal days
7 (P7) and 15 (P15), so comparisons of the development stages could be made. Dissected cochleae were fixed in 4% paraformaldehyde in PBS for 24 hours and placed in 0.1M EDTA to decalcify for 5-10 days. Following decalcification, cochleae were dehydrated in ascending concentrations of ethanol and embedded in methacrylate plastic with the apical surface pointing up. 5µm sections were cut on a motorized microtome, placed on slides, dried, stained with hematoxylin, and viewed under a phase contrast microscope.

**Staining.** Cochlea sections were placed in a series of washes to stain. They were placed in water for two minutes, hematoxylin for fifteen minutes, rinsed in water, Scott’s Tap Water Substitute for five minutes, and lastly rinsed under cold running water to remove any excess Scott’s Tap Water Substitute. Once the slides were stained they were left on a paper towel overnight to air dry. Finally, after being placed on a paper towel to air dry overnight, a strip of Permount mounting medium was added to the slide and the slides were cover slipped.

**Imaging.** Pictures of the stained slides were taken with an Axiostar Microscope and the attached ProgRes C10® camera. The imaging software used was ProgRes® CapturePro. For each cochlea, six pictures were taken of the tectorial membrane, tunnel of Corti, and greater epithelial ridge at the basal, middle, and apical turn.
Data Analyses

Measurements of the total area of tectorial membranes, tunnel of Corti, and GER in the histological sections were measured with ImageJ software at each age group and a one-way ANOVA was used to determine if there was a significant difference in area between genotypes ((Mct8+/+, Oatp1c1+/+), (Mct8+/+, Oatp1c1−/−), (Mct8−/−, Oatp1c1+/+), (Mct8−/−, Oatp1c1−/−)). A post-hoc t-test (Bonferroni) was utilized to determine if there was any significant difference between genotypes.

The amplitude of wave 1 and latencies for peaks 1, 2, and 3 were generated with ABR and measured using SmartEP software. A two-way ANOVA was used to determine if there was any significant difference in amplitudes and latencies between genotypes ((Mct8+/+, Oatp1c1+/+), (Mct8+/+, Oatp1c1−/−), (Mct8−/−, Oatp1c1+/+), (Mct8−/−, Oatp1c1−/−)). A post-hoc t-test (Bonferroni) was utilized to determine if there was any significant difference between genotypes.
Results

A – Pup Weights

Figure 7 summarizes the mean weights for animals sacrificed at P7 or P15. A one-way ANOVA indicated no significant differences were found between genotypes for either age group.

![Figure 7. The effect of genotype on pup weight on postnatal day 7 and 15. Bars represent mean weights ± standard errors. No significant effect of genotype on pup weight was observed.](image)

B. – Cochlear Histology

Figure 8. The qualitative effect of genotype on cochlear development at postnatal day 7 and postnatal day 15 pups. Histological sections of different Mct8/Oatp1c1 genotypes at postnatal day 7 and postnatal day 15 with marked tectorial membrane (TM), tunnel of Corti (black arrow with tail), greater epithelial ridge (ger), inner hair cells (white arrowhead), and outer hair cells (black arrowhead).
Figure 9. The quantitative effect of transporter genotype on cochlear development at postnatal day 7 and postnatal day 15 pups. The effect of genotype on tectorial membrane area of postnatal day 7 and 15 pups (A). The effect of genotype on tunnel of Corti area of postnatal day 7 and 15 pups (B). The effect of genotype on greater epithelial ridge area of postnatal day 7 and 15 pups (C). Bars represent mean area ± standard errors. No significant differences were found in the area of the tectorial membrane, tunnel of Corti, or greater epithelial ridge at P7 or P15. Measurements were taken with ImageJ software.
B.1 – Tectorial Membrane Area

Figure 8 shows histology of a basal turn of the cochlea in postnatal 7 and 15 day old mice with different Mct8 and Oatp1c1 genotypes. Figure 9A summarizes the mean area of the tectorial membrane in the basal, middle, and apical turns of a cochlea in postnatal 7 and 15 day old mice with different Mct8/Oatp1c1 genotypes. A one-way ANOVA was performed for each turn of the cochlea to determine whether there was a significant effect of genotype or age on the development. No significant differences were found between genotypes at either age.

B.2 – Tunnel of Corti Area

Figure 8 shows histology of a basal turn of the cochlea in postnatal 7 and 15 day old mice with different Mct8 and Oatp1c1 genotypes. Figure 9B summarizes the mean area of the tunnel of Corti in the basal, middle, and apical turns of a cochlea in postnatal 7 and 15 day old mice with different Mct8/Oatp1c1 genotypes. A one-way ANOVA was performed for each turn of the cochlea to determine whether there was a significant effect of genotype or age on the development. No significant differences were found between genotypes at either age.

B.3 – Greater Epithelial Ridge Area

Figure 8 shows histology of a basal turn of the cochlea in postnatal 7 and 15 day old mice with different Mct8 and Oatp1c1 genotypes. Figure 9C summarizes the mean area of the greater epithelial ridge in the basal, middle, and apical turns of a cochlea in postnatal 7
and 15 day old mice with different Mct8/Oatp1c1 genotypes. A one-way ANOVA was performed for each turn of the cochlea to determine whether there was a significant effect of genotype or age on the development. No significant differences were found between the genotypes at either age.

C. – Auditory System

C.1.1 – ABR Thresholds

The functional requirement for Mct8 and/or Oatp1c1 was investigated in mutant mice using ABR. Figure 10 shows waveforms generated by a wildtype and a Mct8/Oatp1c1 double KO mouse at 32kHz; these waveforms are representative of both single KO genotypes that were used to determine thresholds, latencies, and wave 1 amplitudes. Figure 11 summarizes the mean auditory thresholds for each genotype at three pure tone frequencies – 8kHz, 16kHz, and 32kHz. One-way ANOVA indicated a significant effect of genotype at 8kHz (F=4.96, P=0.0069) and 32kHz (F=8.69, P=0.0003). Post-hoc analysis demonstrated that ABR thresholds were significantly lower for Mct8 KO compared to wildtype or Oatp1c1 KO. At 32kHz, ABR thresholds were significantly elevated in double KO compared to wildtype and or single Oatp1c1 KO.
Figure 10. Representative waveforms collected from a wildtype and double KO mouse at varying decibels produced by ABR equipment with a pure tone stimulus at 32kHz. Waves I, II and III are labeled with markers at their peaks. These waveforms are representative of the single KO genotypes that were tested.

Figure 11. The effect of genotype on auditory function. Bars represent mean auditory thresholds ± standard error. Statistical analysis demonstrated that mice lacking Mct8 KO had significantly lower ABR thresholds compared to wildtype and Oatp1c1 KO at 8kHz and that double KO had significantly elevated ABR thresholds compared to wildtype and Oatp1c1 single KO at 32kHz. * represents P<0.05 and *** represents P<0.001 using Bonferroni’s t-test n=8 per group.
C.2 – Wave 1 Amplitudes

ABR wave amplitudes are the summation of neurons fired following sound presentation. Assessing amplitude values at specific frequencies and sound intensities provides quantitative methodology for analyzing cochlear output.

Figure 12. The effect of transporter genotype on ABR wave 1 amplitude. Bars represent mean wave 1 amplitudes ± standard error at 8kHz (A), 16kHz (B), and 32kHz (C). n=8 for each Spl and genotype. Note that the electrical change decreases with each 10dB decrease in sound level. c, d, e, and f represent a significant difference between Mct8 KO and double KO, wildtype, and Oatp1c1 KO, respectively, while f represents a significant difference between wildtype and Oatp1c1 KO. Blue circles represent wildtype, green squares represent Oatp1c1 KO, orange upright triangles represent Mct8 KO, and red upside-down triangles represent double KO.

C.2.1 – 8kHz

Figure 12A summarizes the mean wave 1 amplitudes at 8kHz for different Spl and genotypes of Mct8/Oatp1c1. A two-way ANOVA, using genotype and decibel as factors, indicated an effect of Spl (F=34.90, P<0.001), but no effect of genotype or an interaction between the two factors.

C.2.2 – 16kHz

Figure 12B summarizes the mean wave 1 amplitudes at 16 kHz at different Spl and genotypes of Mct8/Oatp1c1. A two-way ANOVA, using genotype and decibel as factors,
indicated no effect of Spl or genotype but identified an interaction between the two factors (F=2.73, P=0.0077). At 100dB, wave 1 amplitudes of Oatp1c1 KO were significantly elevated compared to wildtype.

C.2.3 – 32kHz

Figure 12C summarizes the mean wave 1 amplitudes at 32 kHz for different Spl for Mct8/Oatp1c1 genotypes. A two-way ANOVA was performed to compare genotypes at each Spl level and demonstrated a significant effect of genotype (F= 14.2, P<0.0001), Spl (F=24.8, P<0.0001), and an interaction (F=9.74, P<0.0001) between the two factors. Bonferroni’s post-hoc analysis indicated that wave 1 amplitudes of Mct8 KO at 100dB were significantly elevated compared to wildtype, Oatp1c1 KO, and double KO. At 90dB, wave 1 amplitudes of Mct8 KO were significantly elevated compared to Oatp1c1 KO.

C.3 – Wave Latencies

C.3.1.1 – 8kHz

Figure 13 summarizes the mean wave 1, 2, and 3 latencies at 8kHz for different Spl and Mct8/Oatp1c1 genotypes. A two-way ANOVA was performed for each wave to determine whether there was a significant effect of genotype, Spl, or an interaction. An effect of Spl was observed for all waves (F=277.5, P<0.0001; F=369.5, P<0.0001; F=218.8, P<0.0001; for waves 1, 2, 3 respectively). An effect of genotype and an interaction was observed for waves 2 and 3 (Genotype: F=8.134, P<0.0005; F=12.55,
P<0.0001; Interaction; F=2.626, P=0.01; F=3.104, P=0.003) respectively. Post-hoc analysis demonstrated double KO replicates produced significantly slower latencies than wildtype at 100 and 90 decibels for wave 1. No significant differences were demonstrated at 80 or 70 decibels. For wave 2, double KO latencies were significantly slower than wildtype, Oatp1c1 KO, and Mct8 KO at every Spl. Similarly, for wave 3, double KO latencies were significantly slower than wildtype, Oatp1c1 KO, and Mct8 KO at every Spl.

Figure 13. The effect of transporter genotype on wave latencies at 8kHz. Mean latencies of different genotypes ± standard error of wave 1 (A), wave 2 (B), and wave 3 (C). n=8 for each Spl and genotype. Note that the electrical change decreases with each 10dB decrease in sound level. a, b, and c represent a significant difference between double KO and wildtype, Oatp1c1 KO, and Mct8 KO, respectively. Blue circles represent wildtype, green squares represent Oatp1c1 KO, orange upright triangles represent Mct8 KO, and red upside-down triangles represent double KO.

C.3.1.2 – 16kHz

Figure 14 summarizes the mean wave 1, 2, and 3 latencies at 16kHz for different Spl and Mct8/Oatp1c1 genotypes. A two-way ANOVA was performed for each wave to determine whether there was a significant effect of genotype, Spl, or an interaction. An effect of Spl was observed for all waves (F=426.2, P<0.0001; F=274.5, P<0.0001; F=264.1; P<0.0001; for waves 1, 2, and 3 respectively). An effect of genotype and was
observed for all waves and an interaction observed for wave 1 (Genotype: F=15.71; P<0.0001; F=9.24, P=0.0002; F=0.10, P=0.0002; Interaction: F=9.30, P<0.0001) respectively. For wave 1, a post-hoc analysis demonstrated double KO replicates produced significantly slower latencies than wildtype and Oatp1c1 KO at every Spl; and Mct8 KO replicates demonstrated significantly slower latencies than wildtype, at all every Spl, and Oatp1c1 at every Spl, except for 70 decibels. For waves 2 and 3, double KO replicates demonstrated significantly slower latencies than wildtype and Oatp1c1 KO at every Spl, and Mct8 KO at every Spl except for 70 decibels.

Figure 14. The effect of transporter genotype on wave latencies at 16kHz. Mean latencies of different genotypes ± standard error of wave 1 (A), wave 2 (B), and wave 3 (C). n=8 for each Spl and genotype. Note that the electrical change decreases with each 10dB decrease in sound level. a, b, and c represent a significant difference between double KO and wildtype, Oatp1c1 KO, and Mct8 KO, respectively, d represents a significant difference between wildtype and Mct8 KO, and e represents a significant difference between Oatp1c1 KO and Mct8 KO. Blue circles represent wildtype, green squares represent Oatp1c1 KO, orange upright triangles represent Mct8 KO, and red upside-down triangles represent double KO.

C.3.1.3 – 32kHz

Figure 15 summarizes the mean wave 1, 2, and 3 latencies at 32kHz for different Spl and Mct8/Oatp1c1 genotypes. A two-way ANOVA was performed for each wave to determine whether there was a significant effect of genotype, Spl, or an interaction. An
effect of Spl was observed for all waves (F= 102.2, P<0.0001; F=133.7, P<0.0001; F=186.3, P<0.0001; for waves 1, 2, and 3 respectively). An effect of genotype and interaction was observed for all waves (Genotype: F=16.3, P<0.0001; F=9.1, P=0.0002; F=18.1, P<0.0001; Interaction: F=3.41, P=0.0013; F=5.2, P<0.0001; F=5.9, P<0.0001). Post-hoc analysis demonstrated double KO replicates produced significantly slower latencies than wildtype, Oatp1c1 KO, and Mct8 KO at every Spl for each wave.

Figure 15. The effect of transporter genotype on wave latencies at 32kHz. Mean latencies of different genotypes ± standard error of wave 1 (A), wave 2 (B), and wave 3 (C). n=8 for Spl and genotype. Note that the electrical change decreases with each 10dB decrease in sound level. a, b, and c represent a significant difference between double KO and wildtype, Oatp1c1 KO, and Mct8 KO, respectively. Blue circles represent wildtype, green squares represent Oatp1c1 KO, orange upright triangles represent Mct8 KO, and red upside-down triangles represent double KO.
Discussion

It is widely accepted that proper levels of thyroid hormone are required for the development and maturation of many systems of the body, including the auditory system. This idea is supported by a large body of literature that associates untreated congenital hypothyroidism with a range of developmental problems that range from mental retardation to impaired hearing. Although it was recently demonstrated that human mutations in the MCT8 gene result in severe neurological deficits, offering support that TH transporters are necessary for normal TH-mediated development, whether such transporters are required for auditory system development is unknown. Considering this, the focus of the present study was to further understand the role that thyroid hormone transporters, specifically Mct8 and Oatp1c1, play in the development of the auditory system.

A.1 – Cochlear Histology

The first part of our study investigated a few key cochlear structures essential for normal hearing. Moreover, the selected endpoints are well-described targets of TH action during cochlear development. Therefore, to determine if cochlear development requires TH transporters Mct8 and Oatp1c1, we quantified the structures described below in animals with targeted deletion in Mct8 and/or Oatp1c1.

The GER is a transient cellular mass that regresses and eventually disappears during development leaving an open space medial to the organ of Corti called the spiral (inner) sulcus, which is used as a marker for the onset of hearing (35). Moreover, GER cells
secretes the extracellular proteins that comprise the tectorial membrane (36). In euthyroid mice, the GER completely regresses in 12-14 days. Developmental hypothyroidism delays the regression process with GER cells observable for up to 30 days. In hyperthyroid mice, GER regression is advanced, disappearing in fewer than 8 days (37,38). Analysis of GER area in Mct8 and Oatp1c1 mice indicated no significant differences in the GER area suggesting that Mct8 and Oatp1c1 are not required for TH-mediated GER regression (Figure 8C).

The tectorial membrane is essential for hearing as the deflection of stereocilia that generates action potentials in inner and outer hair cells requires stereocilia embedding within the tectorial membrane. In hypothyroid animals, the tectorial membrane is enlarged, malformed, and often does not come into contact with the hair cells; preventing generation of action potentials and thus impairing hearing (37). In hyperthyroid mice, the tectorial membrane development is advanced resulting in a thin structure due to the premature disappearance of the cells, the greater epithelial ridge (GER), that produce the tectorial (38). In the present study, no significant difference in tectorial membrane area between genotypes was observed (Figure 8A). This finding is consistent with our observation that the regression of the GER is normal in Mct8/Oatp1c1 single and double knockout mice and further supports the idea that Mct8 and Oatp1c1 transporters are largely dispensable for cochlear development.

The tunnel of Corti is a physical separation between inner and outer hair cells formed by specialized cells called pillar cells. Although function of the tunnel of Corti is largely
unknown, it is clear that mice that fail to form this structure have reduced auditory function (39). The opening of the tunnel of Corti is delayed in hypothyroid mice (37), which may contribute to hypothyroidism-induced deafness (40). Conversely, in hyperthyroid mice the tunnel of Corti opens prematurely, thus having a larger area compared to euthyroid mice of the same age (41). These findings indicate that TH controls the timing of tunnel of Corti opening. In the present study, no significant differences were found in the area of the tunnel of Corti between genotypes (Figure 8B). This observation suggests normal development and further supports the idea that TH action within cochlea of mice lacking Mct8 and/or Oatp1c1 is normal.

Although no histological or developmental defects were observed in the cochlea of mice lacking Mct8 and/or Oatp1c1, functional hearing deficits may still exist. For example, the cochlea and its ability to receive sound might be normal, but the central processing might be perturbed. Considering this, auditory function was analyzed by measuring auditory evoked brainstem responses.

B.1 - Physiological Function

Based on histology, the structural integrity of the cochlea appears normal. Therefore, ABR analysis was used to determine what, if any, physiological effect a Mct8 and/or Oatp1c1 deficiency may have on the auditory pathway. The auditory pathway was investigated by measuring ABR hearing thresholds, wave amplitude, and waveform latencies. These tests were performed at different sound frequencies and sound pressure levels to determine if specific areas of the cochlea were affected.
The first physiological test performed was auditory thresholds. Auditory thresholds are important because they indicate how well a mouse can detect sound. At 8kHz, wildtype and Oatp1c1 KO had hearing thresholds that were significantly higher than Mct8 KO (Fig. 7). No significant differences were found at 16kHz, but, interestingly, at 32kHz the hearing thresholds of double KO mice were elevated compared to both wildtype and Oatp1c1 KO mice. While it’s not immediately clear why some KO mice have improved hearing at certain frequencies while other KO mice have impaired hearing it does not appear that defects in cochlear morphology contribute to these deficits; considering the present analysis of the cochlear histology shows no significant difference between the genotypes (Fig. 5). Furthermore, serum THs do not appear to be involved as Mct8/Oatp1c1 double KO mice produce a significantly higher hearing threshold than Mct8 single KO at 32kHz but based on the findings that animals have similar serum TH levels (42). However, it is possible there may be subtle differences in other structures that we did not analyze, like the hair cells or pillar cells, which are necessary for normal auditory function and are known to be effected by hypothyroidism (35).

Based off of the ABR waveform, the amplitude of peak 1, which corresponds to the cochlear nerve, can be measured. Wave amplitude is associated with the strength of the electrical signal generated by the spiral ganglion neurons and thus indicates cochlear output. At the frequency of 8kHz and 16kHz no significant differences were observed, which suggests that the strength of the signal being generated is the same regardless of expression of Mct8 and/or Oatp1c1. At 32kHz, the highest tested frequency, the amplitude for Mct8 KO at 100dB was significantly higher than wildtype, Oatp1c1 KO,
and double KO while at 90dB Mct8 KO was significantly higher than Oatp1c1 KO. Despite their similar TH serum levels, it is not immediately clear why Mct8 single KO mice produced an elevated wave 1 amplitude while Mct8/Oatp1c1 double KO did not. One hypothesis is that Mct8 KO mice have a disrupted olivocochlear system, which plays a protective role in the auditory system by suppressing the sound wave amplification of the outer hair cells. If the olivocochlear system is compromised there would be no limit on the sound wave amplification produced by the outer hair cells which would increase the number of neurons that produce a signal thus increasing the amplitude of wave 1 (43). Overall, with the exception of the Mct8 KO at 32kHz, the wave 1 amplitudes appear to be consistently normal regardless of the expression of Mct8 and/or Oatp1c1 transporters; this suggests the neurons that output from the cochlea are functioning normally.

To understand whether the speed of the neurological signal was being affected by genotype; we extracted wave latencies from the ABR waveforms. These peaks are important because peaks 1 and 2 originate from the cochlear nerve and wave 3 originates from the cochlear nucleus, which is a transition point between the cochlear nerve and the brainstem. Latency was measured from the time an electrical signal was generated to the peak of a wave, which indicates the speed of the electrical signal that is being transmitted. Significant differences between wildtype and single KO (Mct8 or Oatp1c1) mice were only seen at 16kHz. The simplest explanation for this observation is that because 16kHz is most sensitive frequency, and thus, subtle differences in the CNS caused by the single KO’s may have become easier to detect. Interestingly, the data we collected consistently showed, regardless of frequency or Spl, that wildtype mice
generated the fastest latency times and double KO mice generated the slowest latency times. Not only were the double KO latencies consistently the slowest, they were significantly slower than the wildtype mice at every frequency.

A recent study by Mayerl et al. reported T3 and T4 levels in the brain of Mct8 and Oatp1c1 single KOs, or Mct8/Oatp1c1 double KOs. Their findings showed that knocking out Mct8 lowered both T3 and T4 content in the brain, knocking out Oatp1c1 decreased only T4 and knocking out Mct8 and Oatp1c1 dramatically lowered both T3 and T4, which suggests both are necessary for TH to cross the blood-brain-barrier and are needed to maintain TH homeostasis in the CNS (42). Paired with our findings, this suggests that both T3 and T4 are needed to properly develop the auditory pathway in the CNS. This claim is supported by a study performed by Knipper et al. which showed thyroid hormone is important in oligodendrocyte gene expression. Considering these reports, a simple explanation for slower latencies is that in the hypothyroid brain of Mct8/Oatp1c1 double KO mice the axonal fibers that carry auditory information are hypomyelinated. Additionally, their findings showed that TH is necessary for oligodendrocytes to myelinate the auditory axons of the CNS (44), which supports our findings of a functional auditory pathway but slower latencies in the hypothyroid Mct8/Oatp1c1 double KO mice. However, whether the auditory pathway is actually hypomyelinated in Mct8/oatp1c1 double KO mice remains to be determined. An additional analysis of peak-to-peak latencies has yet to be performed, but will be helpful in determining whether the delay in latencies is consistent throughout the auditory pathway or only at the beginning of the auditory pathway.
It is also important to understand that the peripheral nervous system, which includes the inner ear, and the central nervous system develop independently from one another because they originate from different embryonic tissues (45). This difference likely results in different mechanisms for the transport of THs through different tissues. This is important to our study because it explains how the structure of the cochlea was preserved from hypothyroid malformations while the CNS showed a significant delay in latencies, which suggests a possible hypothyroid phenotype.

In summary, based on statistical analysis, our hypothesis can only be partially accepted. We did not observe histological changes in mice deficient in transporter activity, but higher hearing threshold in double KO mice as compared to wildtype mice, at high frequency, were observed. Furthermore, the present study documented the necessity for TH transport for central auditory processing. Based on these experiments we cannot definitively say that hypomyelination is the cause of the observed increased latency, but a strong argument can be made based on prior research that indicates Mct8 and Oatp1c1 are expressed in the blood-brain-barrier, Mct8 and Oatp1c1 double KO mice have drastically decreased brain TH levels, and hypomyelination in many brain regions (42). Based on histology, the cochlea appears to be “euthyroid”; an idea also supported by the observation that ABR thresholds and amplitudes are largely normal. If the cochlea is euthyroid in the absence of Mct8 and/or Oatp1c1, it would be predicted that the Schwann cells, also part of the peripheral nervous system and a known target of TH (44), and their function would also be normal. Unfortunately, the present study did not extend to investigating myelination in the cochlea or the central nervous system.
There are still many questions to be answered about the effects of TH and the role of transporters in the auditory pathway. Based on the present findings, an interesting project in the future could use in situ hybridization to look at the effect that thyroid deficient Mct8/Oatp1c1 double KO mice have on the myelination of the PNS and CNS to determine the origin of the increase in latencies. Further research could also be performed on human patients with Allan-Herndon-Dudley Syndrome by testing their hearing thresholds, amplitudes and latencies to see if they are consistent with our findings in Mct8/Oatp1c1 KO mice since it has been shown they have similar TH serum levels (42).
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