



# The Induction of Hypothyroidism during Gestation Decreases Outer Hair Cell Motility in Rat Offspring

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## Abstract

**Introduction** Perinatal hypothyroidism has a negative repercussion on the development and maturation of auditory system function. However, its long-term effect on auditory function remains unsettled.

**Objective** To evaluate the effect of prenatal hypothyroidism on the auditory function of adult offspring in rats.

**Methods** Pregnant Wistar rats were given the antithyroid drug methimazole (0.02% -1-methylimidazole-2-thiol- MMI) in drinking water, ad libitum, from gestational day (GD) 9 to postnatal day 15 (PND15). Anesthetized offspring from MMI-treated dams (OMTD) and control rats were evaluated by tympanometry, distortion product otoacoustic emission (DPOAE), and auditory brainstem response (ABR) at PNDs 30, 60, 90, and 120.

**Results** Our data demonstrated no middle ear dysfunction, with the OMTD compliance lower than that of the control group. The DPOAE revealed the absence of outer hair cells function, and the ABR showed normal integrity of neural auditory pathways up to brainstem level in the central nervous system. Furthermore, in the OMTD group, hearing loss was characterized by a higher electrophysiological threshold.

**Conclusion** Our data suggest that perinatal hypothyroidism leads to irreversible damage to cochlear function in offspring.

## Keywords

- ▶ congenital hypothyroidism
- ▶ cochlea
- ▶ hearing loss

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## Introduction

The diagnosis of hearing loss during childhood can cause several disturbances in the quality of life, which may include changes in the social, educational, emotional, and language behaviors.<sup>1</sup> Among the many causes of hearing loss, congenital hypothyroidism stands out as a factor of great importance.<sup>2,3</sup> Early diagnosis of the low maternal level of thyroid hormones (THs) allows their replacement and may avoid fetal and subsequent adult damages. Importantly, it is a disease that can be prevented and treated.<sup>4</sup>

Experimental studies have shown the repercussion of hypothyroidism during gestation in the organ of hearing when the hormonal replacement is not done.<sup>2,5</sup> Particularly, abnormalities in the organ of Corti have been reported as some of the main consequences of the lack of THs.<sup>2,3</sup> Congenital hypothyroidism may also be experimentally induced when methimazole, an inhibitor of TH production, is added to the drinking water of pregnant and lactating rats.<sup>3,6</sup>

Thyroid hormone receptors are also of paramount importance to the organ of hearing development during gestation, particularly to the development of the middle and inner ear (ear ossicles<sup>7</sup>) and the organ of Corti.<sup>2,6</sup> In hair cell-specific mutation of TR $\alpha$ 1 or deletion of TR $\beta$  using the Cre-loxP system, hearing procedures such as the measurement of distortion product otoacoustic emission (DPOE) showed impairment of the outer hair cell and auditory brainstem responses (ABRs).<sup>8</sup>

Despite the essential previous studies, there are still many aspects that need to be clarified to provide a better understanding of the impact of prenatal THs on the offspring's auditory system development. In this study, we evaluate the effect of prenatal and postnatal hypothyroidism on the auditory function of adult offspring in rats.

## Material and Methods

### Ethical Approval

All animals and audiological procedures were approved by the Brazilian Ethics Committee on Animal Research (Protocol # 21/2017- CEPA/UFS), following the Brazilian Guide for the Care and Use of Animals in Teaching and Scientific Research Activities (*Diretriz Brasileira para o Cuidado e a Utilização de Animais em Atividades de Ensino ou de Pesquisa Científica - DBCA*), the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publication no. 8023, revised 1978), and the Declaration of Helsinki. All efforts were made to minimize animal suffering and to reduce the number of animals used.

### Animals and the Induction of Gestational Hypothyroidism

All animals were obtained from the animal care facility of Universidade Federal de Sergipe and maintained in a controlled light/dark cycle (12/12 h) with a room temperature of  $23 \pm 2^\circ$  C. Animals had free access to standard chow (Presence – Nestlé Purina Petcare, St. Louis, MO, USA) and drinking water.

Female Wistar rats (~ 200 g) had their estrus cycle monitored daily by vaginal smears. Once the proestrus phase was

detected, adult males (~ 300 g) were put in female cages for overnight mating. The presence of spermatozoa on vaginal smears on the following morning confirmed gestational day (GD) 0. To induce hypothyroidism, dams were given 0.02% methyl mercaptoimidazole (MMI – Sigma-Aldrich, Saint Louis, MO, USA) in drinking water from GD 9 up to the postnatal day (PND) 15, during lactation, as described by Knipser et al.<sup>6</sup>

The offspring from MMI-treated dams (OMTD) were compared to the corresponding control offspring (offspring from water-treated dams; OWTD). The newborns were sexed on postnatal day (PND) 3, and the litter size was adjusted to 8 pups (4 females and 4 males). Pups were weaned on PND 21, and males and females were separated at weaning. From PND 30, all data obtained were analyzed only in males (OWTD and OMTD).<sup>9</sup> For this study, offspring were arbitrarily classified as either preadult (PND 30) or adult (PND 60–120).

### Hearing Analysis Procedures

The pups were anesthetized with an intraperitoneal (i.p) mixture of ketamine (90 mg/kg) and xylazine (10 mg/kg), and the hearing of offspring was measured on PNDs 30, 60, 90, and 120. All of them were evaluated by baseline otoscopic examination (Welch Allyn Pocket Junior, model 22840, SP, Brazil). Rats with a clean external ear canal and normal eardrum were included in the study to have reliable results. After anesthesia, the animals were placed in a small acoustic measurement box, and environmental noise was kept under 50 decibel sound pressure level (dB SPL). For all hearing procedures, we inserted the earphone into the rat's external ear canal with a neonate probe after the head of the animal was brought to a horizontal position.

### Tympanometry Procedure

Tympanometry was performed with a portable tympanometer (Kamplex/Interacoustics, MT 10, Assens, Denmark) with a probe frequency of 226 hertz (Hz). This test measures middle ear pressure using electroacoustic and manometric measurements. The results were recorded by the air pressure of the ear canal, which corresponds to the peak of the tympanogram. It reveals the measurement of compliance in milliliters (mL) and the pressure in daPa. Moreover, compliance provides an index of the tympanic membrane's mobility. Very high, low, or absent mobility indicates a dysfunctional middle ear.

### Distortion Product Otoacoustic Emission (DPOAE)

The DPOAE measurements were performed using a standard commercial device (Otoread – Interacoustics, Assens, Denmark). Distortion product otoacoustic emission was recorded as distortion product diagrams (DPgrams). The intensity of the primary tones was constant, and the DPOAE data were recorded at different frequencies, ranging from 3 to 12 kHz (3, 4, 6, 8, 10, and 12 kHz). For the DPOAE, the intensity of the primary stimuli was set equivalent to its level (L1 = 65 dB SPL and L2 = 55 dB SPL). The frequencies (f1 and f2) were adjusted to  $f2/f1 = 1.21$ . The noise floor level was measured at a frequency of 50 Hz above the DPOAE frequency, using similar averaging techniques. The evaluation of the DPOAE results was based on the amplitude (DPOAE level),

which should be  $\geq 3$  Db SPL. The average time of the test was 60 seconds for each animal.

### Auditory Brainstem Response (ABR)

Auditory brainstem response measurements were performed using a standard commercial ABR meter (EP25–Interacoustics, Assens, Denmark). The ABR stimulus was initially set to 90 decibels sound pressure (dB SPL), 100 microseconds ( $\mu$ s) click (2–4 kHz) presented at a rate of 26.8% with bandpass filtering (0.015 and 3 kHz). Replicate responses (each representing the averaged responses to 1,024 clicks) were recorded. The responses were differential voltage recordings from subdermal needle electrodes placed in the scalp at the vertex (noninverting electrode), ipsilateral mastoid (inverting electrode), and leg (ground electrode).

The presentation level was attenuated in 20 dB SPL steps from 90 dB SPL up to 30 dB SPL. The lowest stimulus level that elicited a repeatable wave was considered as the threshold. Finally, there is a similarity of electrophysiological and behavioral thresholds in rat experiments.<sup>10</sup> The hearing loss classification was: threshold  $\leq 20$  dB SPL (normal); between 30 and 40 dB SPL (soft); between 45 and 70 dB SPL (moderate); between 80 and 90 dB SPL (severe); and higher than 90 dB SPL (profound).<sup>11</sup>

### Data Analysis

The IBM SPSS, Statistics for windows, version 20.0 (IBM Corp., Armonk, NY, USA) was used for data analysis. Results were expressed as values of marginal mean and mean standard error. Mixed analysis of variance (ANOVA) procedures were used for all main and simple effects tests. When the analysis of variance indicated a significant difference, a posthoc significant difference test (Tukey HSD) was performed. Differences were considered significant at  $p \leq 0.05$ .

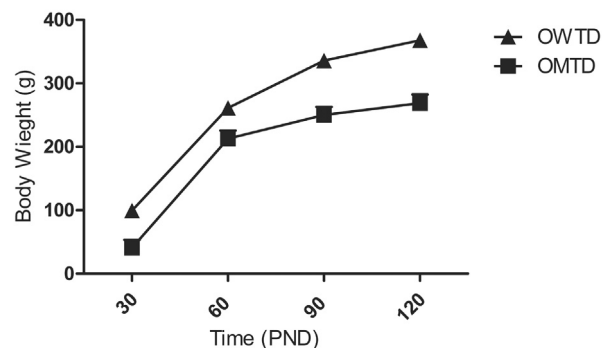
The dependent variable for tympanometry was compliance; for DPOAE, it was amplitude (DPOAE level); and for ABR, it was latency. Random factors included: litter and individual animals, while fixed factors were: treatment during gestation, age, and both ears (right and left).

## Results

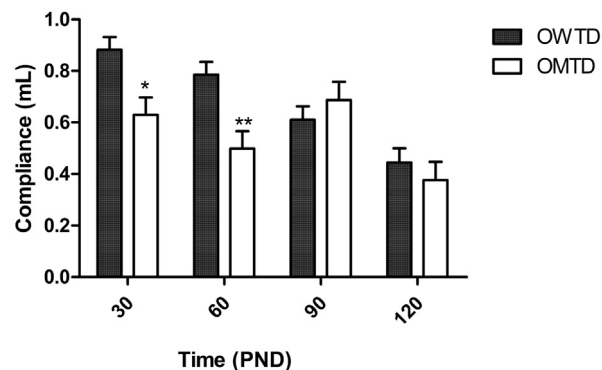
In the analysis of all procedures, the ears were all analyzed together, since there was no significant difference between the right and left ears in the mixed ANOVA ( $p > 0.05$ ).

The evidence that the protocol have ensured hypothyroidism was the OMTD low body mass as shown in ► **Figure 1**.

Tympanometry test results showed a decrease of compliance in OMTD advanced age of analyses as presented in ► **Figure 2**. There was a significant main effect for time factor of OWTD and OMTD [ $F(3; 13.474) = 19.721$ ,  $p < 0.001$ ]. Moreover, there was a significant main effect for experimental groups [ $F(1; 4.397) = 10.368$ ,  $p = 0.028$ ]. The study also revealed a significant interaction between time factor and experimental group [ $F(3; 12.723) = 6.239$ ,  $p = 0.008$ ]. The Tukey honestly significant difference (HSD) posthoc test revealed a statistically significant difference at 30 and 60 days for OMTD ( $p < 0.05$ ).



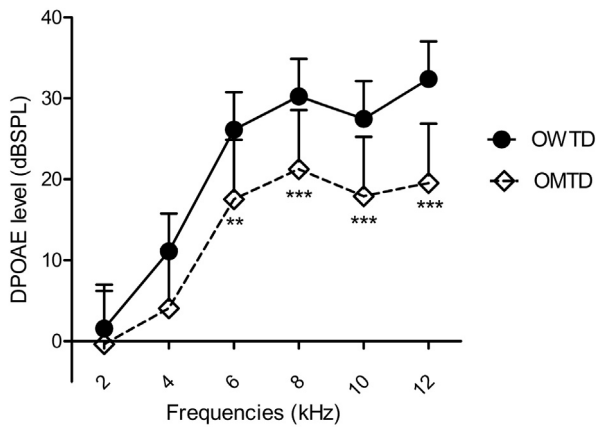
**Fig. 1** Effect of experimental congenital hypothyroidism in offspring body weight at PND 30 (n = 18 OWTD/ n=16 OMTD), PND 60 (n = 18 OWTD/ n = 60 MTD), PND 90 (n = 16 OWTD/ n=5 OMTD), PND 120 (n = 15 OWTD/ n=5 OMTD). For analysis, mixed ANOVA followed by the Tukey HSD post hoc test were used. Values are expressed as marginal mean  $\pm$  SEM. (\*)  $p < 0.05$  and (\*\*)  $p < 0.01$  vs. PND30. PND: Post-natal day, OWTD: Offspring from water-treated dams, OMTD: offspring from MMI treated dams.



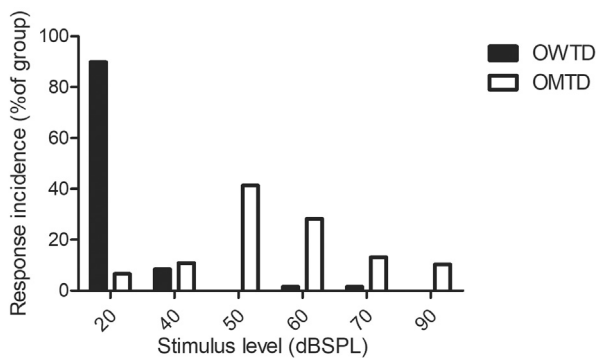
**Fig. 2** Effect of experimental congenital hypothyroidism on compliance in offspring at PND 30 (n = 18 OWTD/ n = 16 OMTD), PND 60 (n = 18 OWTD/ n = 60MTD), PND 90 (n = 16 OWTD/ n = 5 OMTD), PND 120 (n = 15 OWTD/ n = 5 OMTD), as measured by tympanometry. For analysis, mixed ANOVA followed by the Tukey HSD post hoc test were used. Values are expressed as marginal mean  $\pm$  SEM. (\*)  $p < 0.05$  and (\*\*)  $p < 0.01$  vs. PND30. PND: Post-natal day, OWTD: Offspring from water-treated dams, OMTD: offspring from MMI treated dams.

The DPOAE levels (amplitude) for OWTD and OMTD were similar for 2 kHz, with values below 3 dB SPL. The analyses of the OWTD group's amplitudes showed values higher than those in the treated groups, with a significant main effect of experimental groups [ $F(1; 4.519) = 7.762$ ;  $p = 0.043$ ] and time factor [ $F(3; 12.311) = 76.695$ ;  $p = 0.006$ ]. Also, a significant main effect was found for all frequencies [ $F(5; 24.435) = 162.027$ ;  $p = 0.001$ ], and frequencies versus groups [ $F(5; 23.044) = 4.282$ ;  $p = 0.007$ ] (► **Figure 3**). The Tukey's HSD posthoc test revealed a statistically significant difference for frequencies higher than 6 kHz for OMTD ( $p > 0.005$ ).

The 90 dB SPL stimulus were used to analyse ABR absolute latencies and there was no effect of experimental gestational hypothyroidism on latency and absolute waves (peaks I, II, III, IV, and V) in offspring. Therefore, the OWTD and OMTD



**Fig. 3** Effect of experimental gestational hypothyroidism on amplitude and 2, 4, 6, 8, 10, 12 kHz frequencies in offspring ( $n = 18$  OWTD/  $n = 6$  OMTD) as measured with DPOAE test. For analysis, mixed ANOVA followed by Tukey HSD post hoc test. Values are expressed as marginal mean  $\pm$  SEM. (\*\*\*)  $p < 0.001$ . PND: post-natal day, OWTD: offspring from water-treated dams, OMTD: offspring from MMI treated dams, DPOAE: distortion product otoacoustic emission, kHz: kilohertz, dBSPL: decibel sound pressure.



**Fig. 4** Effect of experimental gestational hypothyroidism on incidence of hearing loss in offspring ( $n = 18$  OWTD/  $n = 6$  OMTD) as measured with ABR test. For analysis, mixed ANOVA was used. Values are expressed as marginal mean  $\pm$  SEM. PND: post-natal day, OWTD: offspring from water-treated dams, OMTD: offspring from MMI treated dams, ABR: auditory brainstem response, dB SPL: decibel sound pressure.

groups had the same ABR wave latencies. In contrast, there was an effect of experimental gestational hypothyroidism on the incidence of hearing loss in offspring, once most of the rates in the OMTD group had a hearing threshold up to 40 dB SPL, which is considered a mild-to-profound hearing loss (**Figure 4**). Furthermore, ABR stimulus levels (dB SPL) with a significant main effect for the experimental group were also found [ $F(1; 2.383) = 18.564, p = 0.036$ ].

## Discussion

The induction of hypothyroidism in the animals was confirmed by the low body mass of the rats in the OMTD group, as classically defined in the literature.<sup>12–14</sup> Besides, previous

studies from our laboratory had already shown a reduction in the T3 and T4 serum concentrations in pregnant rats that received similar treatment from GD 9 until the delivery day.<sup>15</sup>

The function of the middle ear was evaluated, analyzing the tympanic ossicular system. Although we found significant differences between the values obtained for OMTD at 30 and 60 days postnatal regarding the age-matched control, all the values obtained were greater than 0.3 mL, thus indicating values of compliance in a normal pattern. These results converge with previous works indicating the integrity of the tympanic ossicular system.<sup>16,17</sup>

In mice, Cordas et al.<sup>7</sup> showed that the lack of maternal THs is essential for the development of the tympanic ossicular system. The activation of thyroid receptors TR $\alpha$ 1 and TR $\beta$  is required in the gestational period, and its absence can delay ossicular maturation, leading to middle ear dysfunction. These data appear to be inconsistent with our results. However, the authors used mutant mice devoid of all known TH receptors that exhibited disorders of the pituitary-thyroid axis, growth, and bone maturation. The complexity of these animals that are unable to respond to any TH is undoubtedly different from those used in our study, in which there was a reduction in T3/T4 levels in perfectly responsive animals. These differences may have been the cause of the differences found in our animals, which, despite lower compliance, did not present functional loss.

Analyzing the values of the untreated animals (OWTD), in **Figure 2**, there is a relationship between compliance and time factor (aging) leading to reduced compliance. Although we have not studied older ages, these values may continue to decline and lead to dysfunction of the middle ear. This idea is supported by physiological changes caused by age as an increase in the stiffness and hardness of the tympanic membrane due to deterioration of the elastin and collagen fibers of the tympanic membrane.<sup>17</sup> The use of an inbred, albino rat strain, the F344, which develops a progressive hearing loss (high frequencies evolving to lower frequencies as the animal ages<sup>9</sup>), did not result in changes in compliance measurement associated with aging, although the data obtained suggested damage to the outer hair cells and a relative hearing and DPOAE loss. The genetic background of this strain, however, can be an essential factor in the differences in sensitivity of the auditory system to TH.

Distortion product otoacoustic emission evaluates the outer hair cells of the inner ear, specifically their electromotility properties.<sup>18</sup> As expected, our data showed the absence of DPOAE response at 2 kHz frequency in both experimental groups. Evidence shows that this result is due to two main aspects; first, the biophysical aspects of the rat auditory system, known as micro ear,<sup>19</sup> and, second, the fact that frequencies lower than 3 kHz generate vertical bands that outer hair cells are unable to respond to.<sup>20</sup> Furthermore, pieces of evidence have been reported showing that this frequency has little or no importance to a rat's auditory system.<sup>21</sup>

We also found an absence of DPOAE response in OMTD. This finding suggests a cochlear dysfunction with reduced or



absent DPOAE amplitude response. These data corroborate previous studies and indicate that the reduced or absent DPOAE amplitude response is due to the decrease of outer hair cell motility. This is a cochlear dysfunction, which can be associated with hearing loss. Several morphological damages were previously reported in the hearing organ when hypothyroidism was induced from DG 10 until PND 14.<sup>2</sup> Abnormalities of the cochlear duct, tectorial membrane, and organ of Corti development were found. The membrane was thicker without hair cells contact. The organ of Corti had a half-turn around the modiolus and a significant delay in development. Hair cells exhibited a reduced number of cells with a smaller size, and irregular appearance. At PND 12, the tectorial membrane was still immature and closely adhering to the internal spiral sulcus. At PND 15, the distortion over the cochlear duct was visible, and the tectorial membrane had no contact with hair cells that displayed longitudinal appearance. Other studies also described the morphological changes of the auditory system in hypothyroidism as tectorial membrane malformation, decreased endocochlear potential, and delay of sensorial epithelium differentiation.<sup>22-25</sup>

Evidence has also shown that hair cells cannot respond to the DPOAE test when morphologically and functionally altered by hypothyroidism during their development. Indeed, Knipper et al.<sup>6</sup> demonstrated that outer hair cells are not capable of electrical and mechanical transduction beyond endocochlear potential and emphasized the importance of THs to the organ of Corti development. These findings indicate that a thyroid hypofunction leads to cochlear damage.<sup>6,8,12</sup>

It is well documented that the longer the induction period of hypothyroidism, the greater the impact on the auditory function will be.<sup>2,6,26</sup> However, the analyses of the auditory neural pathways up to the brainstem level in the central nervous system by ABR revealed the integrity of this via, since no interaction between the experimental groups and wave latencies, and no latency modification were found. Our results, nevertheless, did not agree with those in which increased latency values, mainly for wave I, have been reported when the anti-thyroid drug was given until PND 10,<sup>27</sup> PND 14<sup>6</sup> and PND 30.<sup>28</sup> The differences found in this study may be due to the gestational induction period of hypothyroidism, which does not extend to lactation.

A significant and similar decrease in ABR latency in both groups was found during aging in this study. Studies of Blatchley et al.<sup>29</sup> had already shown the decrease in the amplitude of waves I and II at 8 kHz along the maturational process. Possible changes in amplitude related to the development might include synaptic deficiency from cochlear nuclei to the brainstem, as well as a decrease in the genesis and conduction of electrical potential, which may be implicated in the results we obtained.

In this study, we used wave II as a parameter to determine the electrical hearing threshold.<sup>3</sup> Our data suggest mild-to-profound hearing loss, as consensually described in the literature for congenital hypothyroidism: mild,<sup>30</sup> severe,<sup>28,30,31</sup> and profound.<sup>22</sup> Hearing loss in hypothyroidism

can be explained by the malfunctioning of outer hair cells, such as abnormality in cortical cytoarchitecture and maturational process, impacting mechanical and electrical transduction, and endocochlear potential.<sup>32,33</sup> Moreover, hypothyroidism led to a TR $\beta$  receptor impairment, which conducts to a delay of potassium channel expression that, in turn, affects the endocochlear potential. Abnormalities of afferent dendrites and the absence of efferent innervation of inner hair cells have also been described.<sup>34</sup> In addition, outer hair cells malformation may harm inner hair cells. Receptors  $\alpha$  and  $\beta$  support the mechanical and electrical properties of outer hair cells, so an inadequate control of this system can compromise the function of inner hair cells.<sup>31</sup> Cochlear failure is also a factor of relevance in the auditory system in hypothyroidism. As previously reported, OMTD had a preserved neural integrity of the auditory brainstem with a higher electrophysiological threshold.<sup>8,22,24</sup> However, a more intense stimulus is necessary to evoke a response in ABR when sound transduction in cochlea machinery is deficient.<sup>18</sup>

## Conclusion

In conclusion, this study suggests that perigestational (-gestational/postnatal) exposure to hypothyroidism may reduce the function of the organ of Corti, followed by hearing loss in adult rat offspring, although no changes in the middle ear and neural circuitry up to the brainstem were seen in this study. In summary, the findings reveal lifelong programming of hearing (dys)function evoked by a maternal thyroid disorder during the offspring's intrauterine life.

## Conflict of Interests

The authors declare that there is no conflict of interests.

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