Thyroxine treatments do not correct inner ear defects in *tmprss1* mutant mice

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Abstract

Complete deficiency of a member of the type II transmembrane serine protease family, *tmprss1* (also known as hepsin), is associated with severe to profound hearing loss in mice and a gross enlargement of the tectorial membrane in the cochlea. Levels of thyroxine in these mice have been shown to be significantly lower compared to wild type controls. Since thyroxine is critical for inner ear development, we delivered thyroxine to these mice during the prenatal or postnatal stage of development. Both treatments could not ameliorate hearing loss or correct deformities in the tectorial membrane of these mutant mice, suggesting that a deficiency in *tmprss1* affects thyroxine responsiveness in the inner ear *in vivo*.

Keywords

transmembrane serine protease; hepsin; tectorial membrane; hypothyroidism; cochlea; thyroid hormones; resistance to thyroid hormones

INTRODUCTION

Distinct members of the type II transmembrane serine protease (TMPRSS), *tmprss1* and *tmprss3*, are important for hearing. It is unclear how these proteases contribute to hearing but emerging data have shown that mutations in the *tmprss3* cause non-syndromic congenital and childhood onset autosomal recessive deafness [1,2] whereas a complete deficiency in *tmprss1* in mice leads to hearing loss and inner ear defects [3]. These defects include a grossly enlarged tectorial membrane - an extracellular sheet overlying the organ of Corti with an important role in amplifying low-level signals from outer hair cells; as well as

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a reduced myelination of the auditory nerve [3,4]. These pathologies are also present in hypothyroidism [5,6]. Congenital or drug-induced hypothyroidism is known to affect the proper development and function of the auditory system [4-7]. Because free serum thyroxine (T4) levels in *tmprss1−/−* mice were found to be significantly depressed [3], we administered thyroxine to these mice to determine if it could potentially rescue their auditory deficits.

MATERIALS AND METHODS

Mouse colony

We crossed a male *tmprss1−/−* mouse to a female *tmprss1+/+* mouse to obtain heterozygote offsprings. Matings between these offsprings generate *tmprss1−/−* and *tmprss1+/+* breeders which were used to stock the colony. Mice were genotyped using polymerase chain reaction (PCR) as described [8]. Mice used in this study were approved by the animal ethics committees of the Royal Victorian Eye and Ear Hospital, the Walter Eliza Hall Institute of Medical Research, and the University of Melbourne Gene Technology and Biosafety Committee.

Thyroxine supplementation

For postnatal (P) treatment, newborn *tmprss1+/+* and −/− mice were subcutaneously injected with 50ng T4 (Sigma Aldrich)/g body weight (BW) in 0.9% saline every 2nd day from P2 – P28. In prenatal treatments, 60-day release pellets containing different doses of T4 (5, 15 or 25 mg) or placebo (5, 15 or 25 mg) were purchased from Innovative Research America and implanted subcutaneously in *tmprss1−/−* dams. Implanted dams were mated with *tmprss1−/−* studs 2 weeks later.

Hearing measurements

At 5-6 weeks old, mice were anaesthetised intra-peritoneally with ketamine (75 mg/kg BW; Parnell Laboratories) and xylazil (7.5 mg/kg BW; Troy Laboratories). Computer-generated rarefaction clicks were channelled to a loud speaker positioned 10 cm from the pinna of the measured ear. The non-measured ear was covered with synthetic foam (Otoform, Dreve Otoplastik). Stimuli were presented at a rate of 33 per second and responses were amplified 10^5 fold and bandpass filtered (150 Hz – 3 kHz). Recording typically starts at 100 decibels peak equivalent sound pressure level (dB SPL) and nearer to the threshold; intensity was varied by 5 dB SPL to determine as accurately as possible the auditory brainstem response (ABR) threshold. ABR threshold was determined as the smallest stimulus level required to give peak-trough response amplitude of > 0.3 µV for wave II of the ABR. At each stimulus level, 500 responses were averaged and each recording was repeated to ensure consistency.

Histology

After ABR recordings, mice were sacrificed by CO_2 euthanasia. Cochleae were rapidly dissected, fixed for 2 hrs in 4% paraformaldehyde, decalcified in 10% ethylene diamine tetra-acetic acid (pH7.4), and incubated overnight in 30% sucrose before embedding along the modiolar axis in OCT compound (Sakura). Cochleae were sectioned in 10 µm thickness and stained with haematoxylin and eosin. Cochlear sections separated by a distance of 20-50 µm were examined with a Zeiss Axioplan 2 microscope and a Zeiss Axio Cam camera. Control and treated cochleae in each experiment were treated equally.

Reverse transcription-PCR (RT-PCR)

Age-matched *tmprss1+/+* and −/− cochleae were dissected, snap frozen in liquid nitrogen. RNA isolation and reverse transcription were performed as described [9]. Primers used for

glyceraldehyde-3-phosphate dehydrogenase, *gapdh*, and type II iodothyronine deiodinase, *dio2*, were identical to those previously tested and characterised [9,10]. We used HotStarTaq *Plus* DNA polymerase (Qiagen) and PCR conditions were: denaturation 94°C 3mins; amplification 94°C 1min/54°C 1min/72°C 1min (cycle varied); final extension 72°C 10mins.

RESULTS

It was previously reported that athyroid *pax8−/−* mice have hearing loss and structural abnormalities in the cochlea including a grossly enlarged tectorial membrane; features manifested in *tmprss1−/−* mice [5]. Because early postnatal treatment of T4 could correct the size of the tectorial membrane in *pax8−/−* mice [5], we administered T4 to 11 *tmprss1−/−* and 3 *tmprss1+/+* pups from postnatal day 2 to 28 to determine if postnatal treatment would be sufficient to correct otopathological defects in *tmprss1−/−* mice (Fig. 1). Analysis of at least 5 sections/cochlea from these mice, with section interval of 20-50 μm, showed that *tmprss1−/−* mice receiving T4 continued to show a tectorial membrane similar in size to untreated *tmprss1−/−* mice (Fig. 1C vs B); but the membrane was notably enlarged compared to wild type mice that also received T4 (Fig. 1C vs A). This initial finding prompted us to investigate the use of prenatal T4 treatment in *tmprss1−/−* mice.

Sprenkle et al. (2001) showed that pellets containing T4, purchased from Innovative Research of America, released quantifiable T4 which significantly improved auditory thresholds in congenitally hypothyroid and deaf *hyt/hyt* mice [11]. Therefore, we implanted 3 different doses of T4 (5, 15, 25mg) and their corresponding placebo pellets in *tmprss1−/−* dams before mating to *tmprss1−/−* studs. No pregnancy was achieved in 5 *tmprss1−/−* dams when 25mg T4 pellets were used. For each implanted group, 5-10 pups were randomly selected from at least 2 litters for cochlear histology and ABR measurements. We also included 4 age-matched *tmprss1+/+* mice for comparison. When sections were examined from 5 cochleae in each group, we noticed that the tectorial membranes of all *tmprss1−/−* mice - regardless of treatment - were clearly thicker than *tmprss1+/+* mice (Fig. 2A-D vs 2E). Mean areas and standard deviations (SD) of the tectorial membrane of *tmprss1−/−* mice receiving 5mg T4 or 5mg placebo were 1606 ± 250 μm^2 and 1663 ± 206 μm^2, respectively; and Student's t-test revealed no statistical significance between both groups (Fig. 2F, p=0.7). Although the mean area of the tectorial membrane of *tmprss1−/−* mice receiving 15mg T4 was 1582 ± 235 μm^2, higher than that of the corresponding placebo group (1412 ± 226 μm^2), no statistical significance could be established with Student's t-test (Fig. 2F, p=0.3).

Because *hyt/hyt* mice exposed to prenatal thyroxine treatment showed a significant improvement of hearing thresholds [11], we determined ABR thresholds of *tmprss1−/−* mice receiving T4 or placebo, as well as age-matched *tmprss1+/+* mice. Representative ABR recordings are shown in Fig. 3A-C. Mean ABR threshold and SD of *tmprss1+/+* mice is 63 ± 5 dB SPL (n=8) whereas those of *tmprss1−/−* mice receiving 5mg placebo or T4 were 85 ± 4 dB SPL (n=20) and 84 ± 4 dB SPL (n=14), respectively (Fig. 3D). Increasing T4 dosage failed to improve ABR thresholds since the mean ABR threshold of *tmprss1−/−* mice treated with 15mg T4 remained high at 92 ± 7 dB SPL (n=12), slightly lower than that of the corresponding placebo group, 97 ± 7 dB SPL (n=10). Statistical analysis of ABRs using Student's t-test did not indicate any statistical significance between cohorts receiving 5mg T4 or placebo (Fig. 3D, p=0.6), nor cohorts receiving 15 mg T4 or placebo (Fig. 3D, p=0.09).

Because T4 has to be converted to 3′,3,5-tri-iodothyronine (T3) - the biologically active form - by the cochlear deiodinase gene, *dio2*, we used RT-PCR technique to examine *dio2*

gene expression in *tmprss1−/−* and *tmprss1+/+* mice. We first optimised the number of amplification cycles for *dio2* and *gapdh*, a reference gene, to establish conditions for non-saturating amplification. Next, in 2 experiments using the same number of PCR cycles (30), we fail to observe a diminished level of *dio2* in *tmprss1−/−* mice, relative to *tmprss1+/+* mice (Fig. 4). By amplifying *gapdh* from the same cDNA sample, we noticed that changes of *dio2* are independent of those of *gapdh* (Fig. 4, inset). Therefore, prenatal T4 treatment in *tmprss1−/−* mice does not appear to be compromised by inadequate *dio2* levels in these mice.

Discussion

The discovery that mutations in *tmprss3* cause non-syndromic recessive hearing loss in humans underpins the importance of proteases for hearing [1,2]. However, the mechanism which *tmprss3* influences hearing remains unclear although cell culture studies indicated that it activates epithelial sodium channels which putatively regulate Na^+ levels in the perilymph [12]. Because human temporal bones of deaf individuals affected by *tmprss3* mutations are unavailable, we could not establish if *tmprss3* can be linked to any cochlear pathology. With the availability of *tmprss1* mutant mice, we provided evidence that a deficiency in *tmprss1* induces both abnormalities in tectorial membrane and hearing thresholds [3]. More importantly, the discovery of hypothyroidism in *tmprss1−/−* mice and the indispensable role of thyroid hormones in inner ear development raise a possibility of correcting hearing impairment in these mice with thyroxine treatments [3,13].

Such treatments have been successfully used in mice with mutations in genes that have clearly-defined roles in hypothyroidism [5,11]; for examples, *pax8−/−* athyroid mice which lack thyroid follicles and *hyt/hyt* mice which lack the receptor for thyroid stimulating hormones. In this study, prenatal thyroxine treatment on *tmprss1−/−* mice apparently has no effects in improving hearing thresholds or correcting tectorial membrane abnormality, suggesting that T4 dosage maybe inadequate. This likelihood is ruled out because (i) the dosage of T4 pellets used in this study is either equal or higher than those tested in *hyt/hyt* mice; and (ii) the less severe hypothyroidism in *tmprss1−/−* mice - ~40% less T4 compared to 80% less T4 in *hyt/hyt* mice – would make it more likely for T4 treatment to be effective [11,14].

Because a deficiency in *dio2* expression in mice also causes a grossly enlarged tectorial membrane and raised hearing thresholds [15], it is plausible that a reduction of *dio2* gene expression in *tmprss1−/−* mice can explain our findings. Our RT-PCR experiments show that *dio2* gene expression was not diminished in *tmprss1−/−* mice, indicating that other mechanisms might account for T4 resistance in these mice. Resistance to thyroid hormones (RTH) in humans is commonly attributed to mutations in the thyroid hormone receptor β (TR β) gene [16] but several patients which display clinical symptoms of RTH do not bear mutations in the TR β gene [17], suggesting that other pathways maybe involved. Thus, it is plausible that *tmprss1* may have a role in RTH *in vivo* but further investigation would be necessary to establish the link between *tmprss1* and T4 responsiveness.

Conclusion

Deficiency in *tmprss1* causes a reduction in thyroxine and auditory deficits in mice similar to those in congenital hypothyroidism. Thyroxine treatments fail to correct these deficits suggesting a novel role of *tmprss1* in T4 responsiveness.

Acknowledgments

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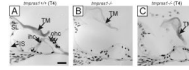
Fig. 1.
Postnatal T4 treatment in *tmprss1+/+* and *tmprss1−/−* mice. Tectorial membrane (TM) in *tmprss1−/−* mice remained enlarged after T4 treatment (C), similar to the untreated *tmprss1−/−* mice (B), but larger than that of *tmprss1+/+* mice receiving identical T4 (A). Abbreviations: SL, spiral limbus; IS, inner sulcus; ihc, inner hair cell; ohc, outer hair cell. Scale bar: 20 μm.
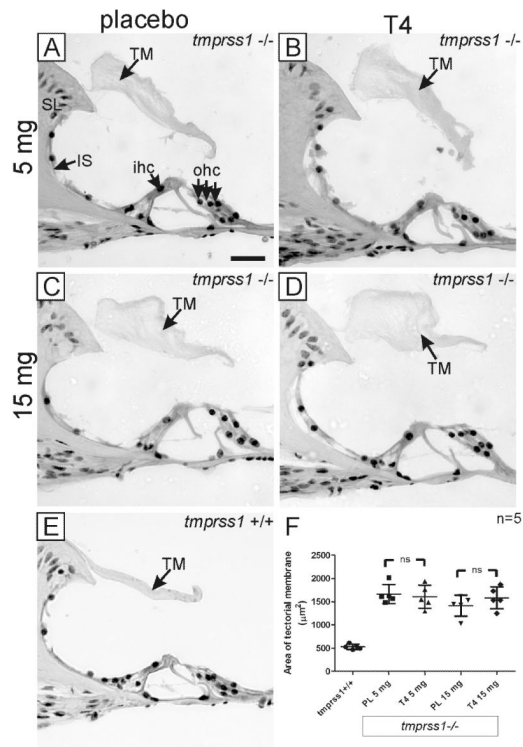
Fig. 2.
Prenatal T4 treatment in *tmprss1−/−* mice and cochlear morphology. Representative cochlear images of *tmprss1−/−* mice receiving 5 mg placebo (A), 5 mg T4 (B), 15 mg placebo (C), 15 mg T4 (D), and a *tmprss1+/+* mouse (E). Scale bar: 20 μm. A graph displaying data point of each area measurement of the tectorial membrane per group, including means and standard deviations, is shown in (F). Abbreviation: ns, not significant.
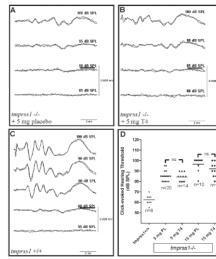
Fig. 3.
Prenatal T4 treatment in *tmprss1−/−* mice and hearing thresholds. Representative ABR recordings of *tmprss1−/−* mice receiving 5 mg placebo (A), 5 mg T4 (B), and a *tmprss1+/+* mouse (C). Threshold of each recording is underlined. A graph displaying data point for each ABR measurement, including means (solid line), is shown in (D).
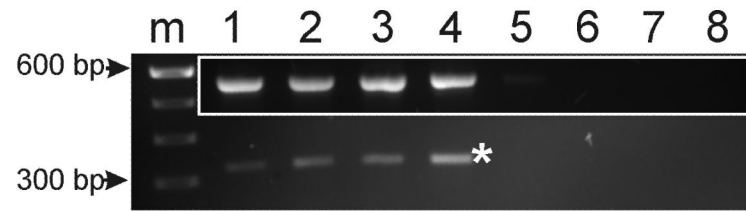
Fig. 4.
RT-PCR of *dio2* and *gapdh* genes in *tmprss1*+/+ and −/− mice. Expression of *dio2* gene (*)
in *tmprss1*+/+ (lanes 1, 3) compared to *tmprss1*−/− (lanes 2, 4) mice. Inset shows *gapdh*
expression from the same cDNA samples. Negative RT-PCR reactions corresponding to
lanes 1-4 are shown in lanes 5-8, respectively. Abbreviation: m, 100 base pair (bp) DNA
markers.