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THYROID HORMONE IS REQUIRED FOR THE PRUNING OF AFFERENT TYPE II SPIRAL GANGLION NEURONS IN THE MOUSE COCHLEA

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Abstract—Afferent connections to the sensory inner (IHCs) and outer hair cells (OHCs) in the cochlea refine and functionally mature during the thyroid hormone (TH)-critical period of inner ear development that occurs perinatally in rodents. In this study, we investigated the effects of hypothyroidism on afferent type II innervation to outer hair cells using the Snell dwarf mouse (*Pit1*^{dw}). Using a transgenic approach to specifically label type II spiral ganglion neurons (SGNs), we found that lack of TH causes persistence of excess type II SGN connections to the OHCs, as well as continued expression of the hair cell functional marker, otoferlin (OTOF), in the OHCs beyond the maturation period. We also observed a concurrent delay in efferent attachment to the OHCs. Supplementing with TH during the early postnatal period from postnatal day (P) 3 to P4 reversed the defect in type II SGN pruning but did not alter OTOF expression. Our results show that hypothyroidism causes a defect in the large-scale pruning of afferent type II SGNs in the cochlea, and a delay in efferent attachment and the maturation of OTOF expression. Our data suggest that the state of maturation of hair cells, as determined by OTOF expression, may not regulate the pruning of their afferent innervation.
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Key words: thyroid hormone, outer hair cells, afferent innervation, type II spiral ganglion neurons, presynaptic marker RIBEYE, peripherin-GFP transgenic mouse.

INTRODUCTION

The inner (IHCs) and outer hair cells (OHCs) in the mature organ of Corti are innervated by the spiral ganglion neurons (SGNs). Mature IHCs are innervated with 90–95% type I SGN fibers (Liberman, 1980). The remaining 5–10% of SGNs are type II unmyelinated neurons that project toward OHC and spiral toward the base to contact multiple OHCs (Liberman, 1980; Brown, 1987). At birth, a mouse cochlea contains an overabundance of type II SGNs. These type II afferent neurons and their fibers make multiple contacts with OHCs, resulting in a surplus number of synapses by the first postnatal week. By the age of onset of hearing, the excess type II SGNs die, and consequently, more than 96% of the OHC afferent synapses are pruned back (Rueda et al., 1987; Echteler, 1992; Barclay et al., 2011). This maturation process takes place during the thyroid hormone (TH) critical period of cochlear development that is in the perinatal period in mice (Deol, 1973; Uziel, 1986; Knipper et al., 1998). An early study by Uziel et al. (1983a) describes defective synaptic pruning in the OHCs of hypothyroid rats, including the persistence of afferent connections that fail to regress normally in the postnatal period. Rueda et al. in another study on rats showed that hypothyroid animals had 22% more total SGNs compared to age-matched controls (Rueda et al., 2003). These studies underscore the importance of TH in the OHC afferent pruning process. However, several important questions remain, such as: (1) What is the effect of hypothyroidism on the synapses of type II SGNs with OHCs? (2) Does supplementing with TH during the early postnatal period restore normal afferent innervation in the hypothyroid OHCs? (3) What is the impact of defective afferent pruning on expression of functionally relevant afferent proteins in the OHC such as otoferlin (OTOF)? (4) Does defective afferent pruning correlate with the delay in OHC efferent innervation? (5) What are the long-term effects of hypothyroidism on OHC synapses? Answers to these questions will provide important insights into the fundamental mechanisms involved in OHC synaptic refinement and maintenance as well as the role of TH in these events.

In an earlier study, we found striking structural defects and functional delays in the pruning of IHC afferent synapses in the Snell dwarf (*Pit1*^{dw}, officially *Pou1f1*^{dw}) hypothyroid mouse model (Sundaresan et al., 2015). In the current study, we focused on the OHCs and examined the role of TH in pruning type II SGNs. We used a transgenic approach to specifically label type II SGNs and count them after the pruning period. We also quantified the number of synaptic ribbons under hypothyroid conditions at different time points during the early postnatal period as well as in the adult mouse. In addition, we

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Abbreviations: ANOVA, analysis of variance; BDNF, brain-derived neurotrophic factor; EDTA, ethylenediaminetetraacetic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, Green fluorescent protein; GLUTR, glutamate receptor; IHC, inner hair cell; MGFP, Mafb-GFP; Ntf3, neurotrophin 3; OHC, outer hair cell; OTOF, otoferlin; PBS, phosphate-buffered saline; PGFP, peripherin-GFP; PTU, propylthiouracil; SGN, spiral ganglion neuron; TEM, transmission electron microscopy; TH, thyroid hormone; WT, wild type.

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examined the ultrastructural pattern of efferent and afferent connectivity to the OHCs and the expression of OTOF, a synaptic vesicle protein involved in neurotransmitter release, under normal and hypothyroid conditions. Furthermore, we established the critical window for TH action on OHC afferent refinement by supplementing *Pit1*^{dw} mice with TH for finite time windows in the early postnatal period. We report that a defect in large-scale pruning of type II SGN is responsible for the abnormal retention of afferent innervation in hypothyroid OHCs.

EXPERIMENTAL PROCEDURES

Mice

The following strains of mice were used in our experiments:

(1) *Pit1*^{dw}: DW/J *Mlph*^{ln} *Pit1*^{dw} /J mice were obtained from the Jackson Laboratory in 1990 and maintained at Stanford University. (2) Peripherin-Green fluorescent protein (PGFP): This transgenic mouse was obtained from Joseph Sarsero, MCRI, Australia. These mice were crossed with the *Pit1*^{dw} strain to generate *Pit1*^{dw}-PGFP mice. (3) Mafb-GFP (MGFP): This strain was obtained from Satoru Takahashi, University of Tsukuba, Tsukuba, Japan. These mice were crossed with the *Pit1*^{dw} strain to generate *Pit1*^{dw}-MGFP mice.

To induce hypothyroidism, pregnant dams were fed with an iodine-deficient diet containing 0.15% propylthiouracil (PTU) (Harlan Labs, Indianapolis, IN, USA, Teklad Animal Diet #95125) throughout the gestation period. Pups were maintained on this diet until the completion of the experiment at which point they were sacrificed. Established procedures for animal care and genotyping were used, including feeding mice a higher fat chow designed for breeding (PMI5020), delaying weaning of mutants until 35 days old, and housing mutants with normal littermates to provide warmth (Karolyi et al., 2007). Control mice were fed normal chow. In all experiments, at least four animals of each genotype were analyzed for each age group studied unless stated otherwise. Postnatal day zero (P0) was designated as the day of birth. All experiments were approved by the University Committee on the Use and Care of Animals and conducted in accord with the principles and procedures outlined in the National Institutes of Health Guidelines for the Care and Use of Experimental Animals.

TH injection

Pit1^{dw} mice and control wild-type (WT) mice were subcutaneously injected for specific durations with either 20 ng of T3/g body weight or phosphate-buffered saline (PBS) as a negative control. T3 solution (20 ng/μl) was prepared by dissolving 1 mg of T3 (3,3,5-triiodo-L-thyronine sodium salt, Sigma Aldrich, St. Louis, MO, USA) in 1 ml 1 N NaOH and 49 ml PBS. Radioimmunoassay was used to confirm that this dose of T3 was sufficient to induce normal T3 levels in *Pit1*^{dw} mice (Sundaresan et al., 2015). Similar studies by other groups have shown that replacement with T3 rather than T4 is more effective since it bypasses the necessary conversion of T4 to the more active T3 *in vivo* (Weiss et al., 1998; Sui et al., 2006). Mice were sacrificed at P14. $n \geqslant 4$ for all treatment groups and controls.

Immunofluorescence staining

Preparation of whole mount cochlear tissues and the procedures for immunostaining on these tissues have been previously described (Mustapha et al., 2009). Inner ears were dissected into cold PBS and after opening the oval and round windows and the bone at the cochlear apex, cochleae were perfused with 4% paraformaldehyde in PBS and were left in fixative solution for an additional 10 min. Samples were washed in PBS for 10 min and then blocked in PBS containing 0.5% Triton X-100 plus 5% bovine serum albumin for 30 min at room temperature. The same blocking buffer was used for diluting antibodies. Primary antibodies were incubated at 4 °C for 36 h followed by three washes in PBS with 0.1% Tween 20. Secondary antibodies were added for 1 h at room temperature, followed by three washes in PBS with 0.1% Tween 20. The following primary antibodies were used: goat anti-CtBP2 polyclonal for RIBEYE (1:200, Santa Cruz Biotechnology, Dallas, TX, USA), rabbit anti-SHANK1 polyclonal (1:200, Neuromics, Minneapolis, MN, USA), rabbit anti-GLUTR2/3 polyclonal for glutamate receptor (GLUTR) (1:200, Millipore, Billerica, MA, USA), rabbit anti-synaptophysin polyclonal (1:300, Pierce Biotechnology, Rockford, IL, USA), and anti-OTOF (1:500, a gift from Drs. Saaid Safieddine and Christine Petit, Pasteur Institute, France). Secondary antibodies used were Alexa Fluor 488-conjugated anti-goat and Alexa Fluor 546-conjugated anti-rabbit (1:500 dilution, Invitrogen). After immunostaining, the cochleae were decalcified in 10% EDTA at room temperature and further fixed by immersion in 4% paraformaldehyde in PBS for 15 min. Cochleae were washed in PBS and mounted on slides in ProLong (Invitrogen) anti-fading media.

Transmission electron microscopy (TEM)

TEM analyses were done as previously described (Mustapha et al., 2009). Briefly, animals were anesthetized and fixed with an intracardiac perfusion of 2.5% glutaraldehyde in 0.15 M cacodylate buffer, pH 7.2 with 0.1% tannic acid. Inner ears were removed and further fixed in the same solution for 2 h. The ears were then decalcified with 3% EDTA with 0.25% glutaraldehyde for 1 week at 4 °C. They were then postfixed in 1% osmium tetraoxide, dehydrated with increasing ethanol concentrations, and embedded in Embed 812 epoxy resin. Embedded ears were sectioned, stained with uranyl acetate and lead citrate, and examined on a Phillips CM-100 TEM. A minimum of four animals per group was examined.

Cochlear RNA extraction and quantitative reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR)

Cochleae were dissected, and RNA extraction and cDNA preparation were performed as previously described (Mendus et al., 2014). To quantify mRNA expression

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