Thyroid hormone receptors $\text{TR}\alpha_1$ and $\text{TR}\beta$ differentially regulate gene expression of $\text{Kcnq4}$ and prestin during final differentiation of outer hair cells

Harald Winter\textsuperscript{1}, Claudia Braig\textsuperscript{1}, Ulrike Zimmermann\textsuperscript{1}, Hyun-Soon Geisler\textsuperscript{1}, Jürgen-Theodor Fränzer\textsuperscript{1}, Thomas Weber\textsuperscript{1}, Matthias Ley\textsuperscript{1}, Jutta Engel\textsuperscript{2}, Martina Knirsch\textsuperscript{2}, Karl Bauer\textsuperscript{2}, Stephanie Christ\textsuperscript{3}, Edward J. Walsh\textsuperscript{4}, JoAnn McGee\textsuperscript{4}, Iris Köpschall\textsuperscript{1}, Karin Rohbock\textsuperscript{1} and Marlies Knipper\textsuperscript{1,*}

\textsuperscript{1}University of Tübingen, Department of Otolaryngology, Tübingen Hearing Research Centre (THRC), Laboratory of Molecular Neurobiology, Elfriede-Aulhorn-Str. 5, 72076 Tübingen, Germany
\textsuperscript{2}University of Tübingen, Institute of Physiology II and Department of Otolaryngology, THRC, Gmelinstr. 5, 72076 Tübingen, Germany
\textsuperscript{3}Max-Planck-Institute for Experimental Endocrinology, Feodor-Lynen-Str. 7, 30625 Hannover, Germany
\textsuperscript{4}Developmental Auditory Physiology Laboratory, Boys Town National Research Hospital, 555 North 30th Street, Omaha, NE 68131, USA

*Author for correspondence (e-mail: marlies.knipper@uni-tuebingen.de)

Summary

Thyroid hormone (TH or T3) and TH-receptor $\beta$ (TR$\beta$) have been reported to be relevant for cochlear development and hearing function. Mutations in the TR$\beta$ gene result in deafness associated with resistance to TH syndrome. The effect of TR$\alpha_1$ on neither hearing function nor cochlear T3 target genes has been described to date. It is also uncertain whether TR$\alpha_1$ and TR$\beta$ can act simultaneously on different target genes within a single cell. We focused on two concomitantly expressed outer hair cell genes, the potassium channel $\text{Kcnq4}$ and the motor protein prestin $\text{Slc26a5}$. In outer hair cells, TH enhanced the expression of the prestin gene through TR$\beta$. Simultaneously $\text{Kcnq4}$ expression was activated in the same cells by derepression of TR$\alpha_1$ aporeceptors mediated by an identified TH-response element, which modulates KCNQ4 promoter activity. We show that T3 target genes can differ in their sensitivity to TH receptors having the ligand either bound (holoreceptors) or not bound (aporeceptors) within single cells, and suggest a role for TR$\alpha_1$ in final cell differentiation.

Introduction

Thyroid hormone (TH or T3) regulates many processes in the development of mammals before the onset of function in a variety of organ systems. This pleiotropic nature of TH is mediated through interactions with ligand-modulated nuclear receptors encoded by two genes TR$\alpha$ and TR$\beta$ (Wu and Koenig, 2000) that can either positively or negatively regulate target genes in response to T3 (Lazar, 2003). To date it is unknown whether T3 target genes can differ in their sensitivity to distinct thyroid hormone receptor (TR) isoforms or to TRs having the ligand bound (holoreceptors) or not bound (aporeceptors). This is a crucial question considering the pathologies associated with hypothyroidism or TR mutations (O’Shea and Williams, 2002). The presumptive redundant effects of coexpressed TRs could definitively mask the individual TR responsiveness of genes and dramatically define the pathological phenotype. To unravel the question of TR specificity at the single-cell level, we focused on two cochlear outer hair cell (OHC) genes that are concomitantly expressed during a crucial time period during which hypothyroidism leads to irreversible hearing deficits in adults (Deol, 1973; Uziel et al., 1985). Neurosensory deafness in humans and rodents is presumed to be related to mutations in the TR$\beta$, leading to resistance to thyroid hormone (RTH), caused by a transdominant negative transcriptional effect (Refetoff et al., 1993; Weiss and Refetoff, 2000). A role of TR$\alpha_1$ for auditory function was suggested in a study of TR$\alpha_2$-deficient mice exhibiting increased expression of TR$\alpha_1$, which was proposed to compensate for missing TR$\beta$ activity (Ng et al., 2001). However, no T3 target genes have been identified in the cochlea at the level of TR isoform-specific transcriptional regulation.

During a developmental period before the onset of hearing, the gene that encodes the outer hair cell motor protein prestin, $\text{Slc26a5}$ (Zheng et al., 2000), is expressed from approximately postnatal day (P) 3 in euthyroid rats, whereas its expression is delayed under conditions of hypothyroidism (Weber et al., 2002). The voltage-dependent K$^+$ channel KCNQ4, which is responsible for the predominant K$^+$ conductance, $I_{K,n}$, of mature OHCs (Marcotti and Kros, 1999) is expressed in rodents from approximately P6 onwards (present study) (Kharkovets et al., 2000). In order to study the specific effect of TH on the expression of these genes, a variety of animal models of hypothyroidism and several TR mutant mice were used to examine their TH dependency. These included (1) animals with goitrogen-induced hypothyroidism; (2) animals that acquired hypothyroidism as a consequence of a naturally occurring point mutation in the thyrotropin receptor (Tshr$^{-agt}$ mutants) (for a review, see Walsh and McGee, 2001); (3)
Both the prestin gene (Weber et al., 2002) and Kcnq4 (present study) were identified as simultaneously expressed T3-dependent genes that are, however, differentially regulated by either TRβ or TRα1. A role for TRα1 in inner-ear development and specifically final differentiation of OHCs was demonstrated. Furthermore, the data show for the first time that apo-TRα1 can repress a T3 target gene independently but in parallel to TRβ acting on a different T3 target gene within the same cell.

### Results

**KCNQ4 and prestin protein expression during early stages of auditory function**

Before the onset of hearing, the subcellular localization of the two outer hair cell proteins KCNQ4 and prestin overlapped between P4 and P7, occupying the entire basolateral membrane area of the OHCs (Fig. 1A, P8). Between P8 and P12, however, the distribution of KCNQ4 gradually shifted to the basal pole of the OHC membrane, whereas prestin shifted to the lateral membrane (Fig. 1A, P12), resulting in a completely separated but neighboring distribution from P12 onwards (Fig. 1B).

**KCNQ4 and prestin protein expression are differentially affected by hypothyroidism**

In the absence of thyroid hormone, the expression of prestin was reduced and the distribution pattern remained immature (Fig. 2A, hypo, red) (Weber et al., 2002). By contrast, KCNQ4 expression was completely lacking in the OHCs of hypothyroid rats (Fig. 2A, hypo, green). Using northern blot analysis both the ~3.9 kb and the ~3.8 kb Kcnq4 transcripts were detected (Fig. 2B, con as) (Kubisch et al., 1999), which were significantly reduced in the cochleae of hypothyroid animals (Fig. 2B, hypo, as). The corresponding sense probes showed no signal (Fig. 2B, con, sense). Reduced Kcnq4 mRNA levels were also noted in the cochleae of P12 hypothyroid rats (Fig. 2C, hypo) compared with control animals (Fig. 2C, con) using semi-quantitative RT-PCR. The housekeeping gene cyclophilin was used as an internal control. In addition, Kcnq4 mRNA was detected by in situ hybridization in both outer and inner hair cells of control animals (Fig. 2D, control) (see also Oliver et al., 2003) and was absent in age-matched hypothyroid rats (Fig. 2D, hypo).

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**Table 1. Mouse mutants used**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Molecular characterization</th>
<th>Pathophysiology</th>
<th>Reference</th>
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<tr>
<td><em>Pax8</em>&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Targeted inactivation of the <em>Pax8</em> gene</td>
<td>Absence of thyroid follicular cells</td>
<td>Christ et al., 2004 Mansouri et al., 1998</td>
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<tr>
<td><em>Tshr&lt;sup&gt;hyt/hyt&lt;/sup&gt;</em></td>
<td>Naturally inherited, autosomal recessive P556L point mutation in the <em>Tshr</em></td>
<td>Primary, congenital hypothyroidism</td>
<td>Beamer et al., 1981 Sprenkle et al., 2001a Sprenkle et al., 2001b Sprenkle et al., 2001c Stein et al., 1994</td>
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<td><em>TRα&lt;sup&gt;1&lt;/sup&gt;−/−</em></td>
<td>Replacement of <em>TRα1</em>-specific coding region with that of <em>TRα2</em></td>
<td>Abnormal heart rate</td>
<td>Wikstrom et al., 1998</td>
</tr>
<tr>
<td><em>TRβ&lt;sup&gt;−/−&lt;/sup&gt;</em></td>
<td>Targeted inactivation of the <em>TRβ</em> gene</td>
<td>Recessive resistance to thyroid hormone Hyperthyroidism Deafness</td>
<td>Forrest et al., 1996b</td>
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<td><em>TRα&lt;sup&gt;1&lt;/sup&gt;−/−</em>&lt;sup&gt;β&lt;sup&gt;−/−&lt;/sup&gt;&lt;/sup&gt;</td>
<td>Compound mutant mice, generated by crossing <em>TRα1</em> and <em>TRβ</em> mice</td>
<td>Hyperthyroidism Retarded growth Retarded bone maturation Female infertility Deafness</td>
<td>Gothe et al., 1999 Rusch et al., 1998</td>
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<td><em>TRα&lt;sup&gt;1&lt;/sup&gt;−/−</em>&lt;sup&gt;m&lt;sup&gt;−/−&lt;/sup&gt;&lt;/sup&gt;</td>
<td>Dominant-negative R384C mutation introduced in <em>TRα1</em></td>
<td>Tenfold reduced ligand binding Retarded postnatal development and growth Cardiac function abnormalities</td>
<td>Tinnikov et al., 2002</td>
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**Fig. 1.** Coincident redistribution of KCNQ4 and prestin in rat OHCs. (A) Immunohistochemistry shows KCNQ4 (green) and prestin (red) in OHCs before (P8) and at onset of hearing (P12). (B) Double immunohistochemistry of prestin (red) and KCNQ4 (green) in a mature (P21) OHC. Arrows, KCNQ4 and prestin protein; arrowheads, basal pole of OHCs. Bars, 20 μm.
In two commonly used mouse models of hypothyroidism, i.e. Tshρ<sup>hyt</sup> mutants (Hyt/Hyt) which have a point mutation in the thyrotropin receptor, and Pax8<sup>−/−</sup> mutants which lack thyroid follicular cells, similar KCNQ4 and prestin expression patterns were observed (data not shown). This verifies the uniformity of TH influence, independent of the mode of induction of hypothyroidism.

KCNQ4 but not prestin protein expression differs depending on the absence of thyroid hormone or its receptors

KCNQ4 and prestin protein expression profiles in the absence of TH were compared with those in TR<sub>α1</sub>−/−/TR<sub>β2</sub>−/− mutant mice, which carry deletions of both TR<sub>α</sub> and TR<sub>β</sub>. Immunohistochemical analyses were carried out with antibodies against KCNQ4 or prestin, along with the efferent-specific marker synaptophysin (Gil-Loyzaga and Pujol, 1988; Knipper et al., 1995), shown for P10 animals in Fig. 3.

Similar to prestin expression profiles in hypothyroid animals (Fig. 3A, red) prestin was expressed in TR<sub>α1</sub>−/−/TR<sub>β2</sub>−/− mutants (Fig. 3C, red), however its distribution remained immature. Surprisingly, in contrast to absent KCNQ4 expression in hypothyroid animals (Fig. 3B, red) KCNQ4 was expressed when both TR<sub>α</sub> and TR<sub>β</sub> were deleted (Fig. 3D, red), although its subcellular distribution was still immature. The results suggest that Kcnq4 gene expression is regulated by a TH aporeceptor that may repress transcription (Perissi et al., 1999), and when deleted, might cause the less-severe KCNQ4 phenotype observed in the TR<sub>α1</sub>−/−/β<sup>−/−</sup> mutants.

KCNQ4 and prestin protein expression is differentially regulated by either TR<sub>α1</sub> or TR<sub>β</sub>

To test this inference, the effect of hypothyroidism in animals with single TR deletions was considered. The TR subtype responsible for repression of Kcnq4 should be identifiable, as its deletion should render the Kcnq4 gene independent of the influence of TH.

KCNQ4 and prestin protein expression was analyzed in both TR<sub>α1</sub>−/− and TR<sub>β2</sub>−/− mutants in which hypothyroidism had been induced by treating neonates with the goitrogen MMI (methylmercapto-imidazol). A subset of goitrogen-treated mutant mice were rescued from hypothyroidism by daily injections of T4 from birth onwards to serve as a control condition. When hypothyroidism was induced in neonatal TR<sub>β2</sub>−/− mutants, KCNQ4 protein was not expressed in OHCs (Fig. 4A, red), whereas prestin was expressed but the immature subcellular distribution persisted (Fig. 4C, red). By contrast, KCNQ4 expression was normal in TR<sub>α1</sub>−/− mutants even under conditions of induced hypothyroidism (Fig. 4B, red), confirming that the TR<sub>α1</sub> deletion promoted the expression of specific marker synaptophysin (Gil-Loyzaga and Pujol, 1988; Knipper et al., 1995), shown for P10 animals in Fig. 3.

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KCNQ4 and prestin protein expression is differentially regulated by either TR<sub>α1</sub> or TR<sub>β</sub>
KCNQ4. In the case of the prestin gene, the deletion of TRα1 did not alter the typical expression profile observed in hypothyroidism; i.e. an immature subcellular distribution pattern persisted (Fig. 4D, red). These findings collectively validate the view that apo-TRα1 represses KCNQ4 expression, but does not affect the expression of prestin.

Treatment with T4 rescued the KCNQ4 phenotype from neonatally induced hypothyroidism in TRβ+/− mutants (Fig. 4E, red), while not affecting the normal phenotype in TRα1−/− mutants (Fig. 4F, red), a finding that is consistent with the idea that TH mediates KCNQ4 expression independently of TRβ activity by releasing the Kcnq4 gene from the repressive action of TRα1. Consistent with the idea that TRβ rather than TRα1 controls prestin expression and distribution, prestin expression is insensitive to TH when TRβ is deleted (Fig. 4G, red) whereas its expression remains dependent on TH in TRα1−/− mutants (Fig. 4H, red). Thus, OHCs express two genes that are simultaneously but differentially regulated by either TRα1 or TRβ.

**Fig. 4.** Deletion of TRα1 but not TRβ exclusively restores KCNQ4 expression in OHCs despite hypothyroidism. (A,C) At P12, in hypothyroid TRβ−/− mutants (hypo), KCNQ4 protein was not observed (A), prestin was expressed but its distribution remained immature (C). (B,D) In hypothyroid TRα1−/− mutants (hypo), KCNQ4 appears normal (B), whereas prestin persists in an immature distribution (D). (E,F) T4-mediated rescue leads to a normal adult expression of KCNQ4 in hypothyroid TRβ−/− mutants (E), like in T4-treated TRα1−/− mutants (F). (G,H) T4 does not rescue prestin from its immature pattern in hypothyroid TRβ−/− mutants (G), but does so in hypothyroid TRα1−/− mutants (H). Sections were co-immunolabeled with synaptophysin (green) and DAPI (blue). Arrows, basal pole of OHC. Bar, 10 μm.

A TRE in the KCNQ4 gene mediates repressive apo-TRα1 activity on the Kcnq4 promoter in the absence of TH in vitro

The data so far clearly indicate a difference in the regulation of prestin and KCNQ4 expression by TRβ or TRα1, respectively. Although TREs in the prestin gene have been described and one, TREPrest, has been functionally characterized (Weber et al., 2002), information about TREs associated with the Kcnq4 gene is currently unavailable. Using MatInspector professional software (release 2.0; http://www.genomatix.de) (Quandt et al., 1995), putative response elements, TREKCNQ4, were identified 3-10 kb upstream of the transcription start site, as deduced by Kubisch et al. (Kubisch et al., 1999) for the human Kcnq4 gene (Fig. 5A). We focused on three identified putative TREKCNQ4 because they contained two hexamers that are almost identical to the consensus sequence AGGTCA. The hexamers are organized as either a direct repeat (TRE-1KCNQ4), a palindrome (TRE-2KCNQ4), or an inverted palindrome (TRE-3KCNQ4) with a 4, 8 and 8 bp spacer, respectively (Fig. 5A).

The protein binding properties of the three TREKCNQ4 were studied using electromobility shift assay (EMSA). Recombinant chicken TRα (cTRα) shifted [32P]TRE-3KCNQ4 (Fig. 5B, lane 4) to similar positions as a 32P-labeled control TRE (DR4; Fig. 5B, lane 1), which correspond to a homodimer complex with a lower electromobility and a monomer complex with a higher electromobility. Neither [32P]TRE-1KCNQ4 (Fig. 5B, lane 2) nor [32P]TRE-2KCNQ4 (Fig. 5B, lane 3) was able to bind cTRα. The shift of [32P]TRE-3KCNQ4 (Fig. 5B, lane 4) was equally diminished whether unlabeled TRE-3KCNQ4 (Fig. 5B, lane 5) or DR4 (Fig. 5B, lane 6) oligomers were added as a competitor. Unlabeled oligomers specific for the transcription factor TFIIID had no effect on this interaction (Fig. 5B, lane 7).

Nucleotide exchanges introduced in the 5′ half-site of the unlabeled TRE-3KCNQ4 (Fig. 5C, TRE-3mut1) used as a competitor had no influence on the capability of cTRα to shift the [32P]TRE-3KCNQ4 (Fig. 5B, lane 8), indicating that competitor function is lost upon mutation of the 5′ half-site. Nucleotide exchanges introduced in the 3′ half-site of TRE-3KCNQ4 (Fig. 5C, TRE-3mut2) still abolished the interaction of cTRα and [32P]TRE-3KCNQ4 (Fig. 5B, lane 9), indicating that this oligomer still functions as a competitor. The results indicate that a TRE upstream of the Kcnq4 promoter is capable of binding TRα.

Using PromoterInspector software (release 2.0; http://www.genomatix.de) (Scherf et al., 2000), a minimal promoter sequence in the 5′ upstream region starting at position −495 bp to +45 bp relative to the start point of transcription of the Kcnq4 gene was identified. The introduction of this sequence into a promotorless reporter gene vector led to a significant 4.3-fold (±0.3 s.d.; n=4) increase in reporter gene activation (Fig. 5D, white column) when compared with the promotorless vector, specifying the minimal promoter of Kcnq4.

A series of transfections were performed to examine the TH responsiveness of the reporter gene expression upon the subcloning of the TRE-3KCNQ4 upstream of the Kcnq4 promoter sequence. The introduction of the TRE-3KCNQ4 upstream of the identified promoter sequence led to a significant decline of the reporter gene activity to 2.1±0.5 s.d. (P<0.05; n=4) with co-transfected TRα in the absence of
ligands (Fig. 5D, black column). The addition of T3 had only a subtle effect on the reduction of the reporter gene activity (data not shown). Only recently, Lee and Privalsky (Lee and Privalsky, 2005) showed that TR-RAR (retinoic acid receptor) heterodimers can also form on different TREs, including inverted palindromes. In contrast to TR-RXR (retinoid X receptor) heterodimers which were shown to recruit only co-activators, TR-RAR dimers can also recruit co-repressors, which are, however, only efficiently released from TR-RXR heterodimers upon the addition of both ligands, T3 and all-trans retinoic acid (ATRA) (Lee and Privalsky, 2005). Indeed, in contrast to adding only T3, the addition of T3 and ATRA led to a significant increase of reporter gene activity (4.1±1.1 s.d.; P<0.05; n=3) to similar levels seen with the KCNQ4 promoter alone (Fig. 5D, compare gray column with white column), indicating an involvement of endogenous RARs.

In line with previous experiments (Yen et al., 1992), we noted that in EMSAs T3 decreased TRα homodimer binding to TRE-3KCNQ4 whereas monomer binding remained unaffected (Fig. 5E).

Both, TRα and TRβ bind to TRE-3KCNQ4 and to TREPrest

To gain further insight into the mechanism of how differential TR activity on two genes is achieved in a single cell, we verified in a first approach the parallel expression of TRα1 and TRβ in OHCs at the time of final hair cell differentiation. To date, evidence of TR expression in OHCs has come from separate studies done at the protein level, for TRα1 (Lautermann and ten Cate, 1997) and TRβ (Knipper et al., 2001). Using a novel technique (Michna et al., 2003) to specifically dissect OHCs from rodents, both TRα1 and TRβ transcripts were detected in isolated OHCs around the time of onset of hearing by RT-PCR (data not shown). In a next step we questioned a preference of TRα or TRβ for either TRE Prest or TRE-3KCNQ4. Using EMSA, in vitro translated rat TRα (rTRα) and TRβ (rTRβ) shifted the [32P]TRE-3KCNQ4 (Fig. 6, lane 1 and 3) and [32P]TREPrest (Fig. 6, lane 5 and 7) to positions comparable to those of cTRα. Unlabeled TRE-3KCNQ4 and TREPrest competitor oligonucleotides specifically blocked the binding of rTRα and rTRβ to [32P]TRE-3KCNQ4 (Fig. 6, lane 2 and 4) and [32P]TREPrest (Fig. 6, lane 6 and 8), respectively, indicating
that both receptors, TRα and TRβ, bind to both TRE-3KCNQ4 and TREprest.

A dominant-negative mutation in the TRα1 receptor causes repression of Kcnq4 gene expression but has no effect on prestin.

The repressive activity of TRα1 on Kcnq4 but not on the prestin gene was confirmed by analyzing a mouse model in which the TRα1 gene expressed a dominant-negative point mutation (R384C) that renders the receptor insensitive to TH (Tinnikov et al., 2002). Genes that are repressed by a dominant-negative mutation (R384C) that renders the receptor insensitive to TH (Tinnikov et al., 2002). Genes that are repressed by rTRα or rTRβ with [32P]TRE-3KCNQ4 is significantly reduced in the presence of an excess of unlabeled TRE-3KCNQ4 competitor oligomers (lanes 2,4). The same is true for rTRα or rTRβ with [32P]TREprest by using TREprest as a competitor (lanes 6,8).

Fig. 6. Comparison of binding properties of TRE-3KCNQ4 and TREprest by EMSA. In vitro translated rat TRα (rTRα) and rTRβ shifts [32P]TRE-3KCNQ4 (lanes 1,3) as well as [32P]TREprest (lane 5,7) to two complexes with different electromobility (filled and open arrowheads). The interaction of rTRα or rTRβ with [32P]TRE-3KCNQ4 is significantly reduced in the presence of an excess of unlabeled TRE-3KCNQ4 competitor oligomers (lanes 2,4). The same is true for rTRα or rTRβ with [32P]TREprest by using TREprest as a competitor (lanes 6,8).

Fig. 7. Kcnq4 and prestin expression in OHCs of heterozygous TRα1m/+ mutants at P13. (A,B) In OHCs of TRα1m/+ wild type, expression and distribution of Kcnq4 (A, red) and prestin (B, red) is normal. (C,D) In OHCs of TRα1m/+ mutants Kcnq4 is completely absent (C) whereas prestin expression and distribution is normal (D). (E,F) By contrast, Kcnq4 expression in vestibular hair cells (VHC) is normal within the same sections of both the wild type (E) and TRα1m/+ mutants (F). Sections were co-immunolabeled with synaptophysin (green) and DAPI (blue). Bar, 20 μm.
In this study we provide evidence that OHCs express at least two T3 target genes, Kcnq4 and prestin, which are differentially regulated by TRα1 or TRβ, respectively. The findings reported here reveal for the first time a role of TRα1 in inner-ear development, and suggest a molecular mechanism for the occurrence of gene repression parallel to the gene expression switch from apo-TRα1 to holo-TRα1. Moreover, a crucial time period is defined during which a switch from apo-TRα1 to holo-TRα1 occurs independently of the presence of TRβ activity, an event that is presumably defined by a local peak of TH level.

TRα1 activity during final differentiation of the inner ear

The abnormal regulation of genes controlled by TRβ has long been presumed to be the cause of profound neurosensory deafness associated with congenital hypothyroidism (Forrest et al., 1996a; Forrest et al., 1996b). Resistance to TH owing to a point mutation within the TRβ gene (Adams et al., 1994; Refetoff et al., 1967; Weiss and Refetoff, 2000) has suggested a crucial role of apo-TRβ in normal developmental processes, including development of hearing. However, genes that are affected by TRβ have not been identified so far. Until now no evidence for a role of TRα in hearing existed (Ng et al., 2001). This may be due to the fact that to date most terminal differentiation processes in the organ of Corti have not been regarded in the context of hypothyroidism.

Apo-TRα1 has recently been shown to exert a repressive influence on gene regulation (Flamant et al., 2002). The unlocking from TRα1 aporeceptor-mediated gene repression appears to become necessary towards the second to third postnatal week in rodents. T3-based compensatory treatments indicate that the brain (Eayrs, 1971; Morte et al., 2002), bone, spleen, intestine (Flamant et al., 2002), heart (Mai et al., 2004) and cochlea (Christ et al., 2004; Knipper et al., 2001) depend on T3 during the second postnatal week. If, however, T3 replacement occurs beyond the second postnatal week, the capacity to rescue hypothyroidism-induced pathology is significantly reduced (Christ et al., 2004; Flamant et al., 2002; O’Shea and Williams, 2002; Tinnikov et al., 2002).

A life-threatening situation resulting from a failure of timely activation of distinct genes repressed by the apo-TRα1 was demonstrated by the early postnatal death of Pax8<sup>−/−</sup> mice, which could be prevented by the deletion of TRα (Flamant et al., 2002). Although recent findings suggest that in addition to TRα1, TRα2 has to be deleted to rescue Pax8<sup>−/−</sup> mice (Mittag et al., 2005), data in the present study (Figs 4, 7) point to a restricted role of apo-TRα1 on Kcnq4. Considering the detrimental influence of a persistent gene repression mediated by TH aporeceptors (Flamant and Samarut, 2003), it will be challenging to investigate whether postnatal death of OHCs in Pax8<sup>−/−</sup> mice (Christ et al., 2004) is due to persistent repressive activity of apo-TRα1 on Kcnq4. This is important in light of the previously reported death of OHCs caused by either a decreased Kcnq4 expression (Rüttiger et al., 2004), a pharmacological blockade of KCNQ4 channels (Nouvian et al., 2003) or deletion of Kcnq4 (Kharkovets et al., 2006).

Specificity of TR-mediated transcriptional control of two concomitantly expressed T3 target genes in a single cell

Based on recent efforts to classify TRs (Chassande, 2003), we suggest that TRβ acts on the prestin gene as a ‘silent’ receptor in the absence of TH (Fig. 8A), whereas TRα1 acts as aporeceptor on Kcnq4. Previously acquired data (Knipper et al., 1999; Lautermann and ten Cate, 1997) and data resulting from the present study showing that both TRα and TRβ are expressed in OHCs at the time of Kcnq4 and prestin expression, exclude the possibility that tissue-specific differences in TR levels define

Discussion

In this study we provide evidence that OHCs express at least two T3 target genes, Kcnq4 and prestin, which are differentially regulated by TRα1 or TRβ, respectively. The findings reported here reveal for the first time a role of TRα1 in inner-ear development, and suggest a molecular mechanism for the occurrence of gene repression parallel to the gene expression switch from apo-TRα1 to holo-TRα1. Moreover, a crucial time period is defined during which a switch from apo-TRα1 to holo-TRα1 occurs independently of the presence of TRβ activity, an event that is presumably defined by a local peak of TH level.

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The abnormal regulation of genes controlled by TRβ has long been presumed to be the cause of profound neurosensory deafness associated with congenital hypothyroidism (Forrest et al., 1996a; Forrest et al., 1996b). Resistance to TH owing to a point mutation within the TRβ gene (Adams et al., 1994; Refetoff et al., 1967; Weiss and Refetoff, 2000) has suggested a crucial role of apo-TRβ in normal developmental processes, including development of hearing. However, genes that are affected by TRβ have not been identified so far. Until now no evidence for a role of TRα in hearing existed (Ng et al., 2001). This may be due to the fact that to date most terminal differentiation processes in the organ of Corti have not been regarded in the context of hypothyroidism.

Apo-TRα1 has recently been shown to exert a repressive influence on gene regulation (Flamant et al., 2002). The unlocking from TRα1 aporeceptor-mediated gene repression appears to become necessary towards the second to third postnatal week in rodents. T3-based compensatory treatments indicate that the brain (Eayrs, 1971; Morte et al., 2002), bone, spleen, intestine (Flamant et al., 2002), heart (Mai et al., 2004) and cochlea (Christ et al., 2004; Knipper et al., 2001) depend on T3 during the second postnatal week. If, however, T3 replacement occurs beyond the second postnatal week, the capacity to rescue hypothyroidism-induced pathology is significantly reduced (Christ et al., 2004; Flamant et al., 2002; O’Shea and Williams, 2002; Tinnikov et al., 2002).

A life-threatening situation resulting from a failure of timely activation of distinct genes repressed by the apo-TRα1 was demonstrated by the early postnatal death of Pax8<sup>−/−</sup> mice, which could be prevented by the deletion of TRα (Flamant et al., 2002). Although recent findings suggest that in addition to TRα1, TRα2 has to be deleted to rescue Pax8<sup>−/−</sup> mice (Mittag et al., 2005), data in the present study (Figs 4, 7) point to a restricted role of apo-TRα1 on Kcnq4. Considering the detrimental influence of a persistent gene repression mediated by TH aporeceptors (Flamant and Samarut, 2003), it will be challenging to investigate whether postnatal death of OHCs in Pax8<sup>−/−</sup> mice (Christ et al., 2004) is due to persistent repressive activity of apo-TRα1 on Kcnq4. This is important in light of the previously reported death of OHCs caused by either a decreased Kcnq4 expression (Rüttiger et al., 2004), a pharmacological blockade of KCNQ4 channels (Nouvian et al., 2003) or deletion of Kcnq4 (Kharkovets et al., 2006).

Specificity of TR-mediated transcriptional control of two concomitantly expressed T3 target genes in a single cell

Based on recent efforts to classify TRs (Chassande, 2003), we suggest that TRβ acts on the prestin gene as a ‘silent’ receptor in the absence of TH (Fig. 8A), whereas TRα1 acts as aporeceptor on Kcnq4. Previously acquired data (Knipper et al., 1999; Lautermann and ten Cate, 1997) and data resulting from the present study showing that both TRα and TRβ are expressed in OHCs at the time of Kcnq4 and prestin expression, exclude the possibility that tissue-specific differences in TR levels define
TR specificity, as previously suggested (Dillmann, 2002). We cannot, however, rule out the fact that differences in the level of either TRα or TRβ in OHCs may play a role in TR specificity. As both TRs were able to bind to both TRE\textsuperscript{Prest} and TRE\textsuperscript{KCNO4} (Fig. 6), we can be certain that the TR specificity seen in the case of Kcnq4 and prestin is not determined by differences in sequence-specific binding properties of the TREs, as previously described (Olson et al., 1998).

Local changes in T3 level should instead be considered to play a crucial role in influencing TR specificity. Indeed, in humans and rodents the critical developmental time period of the inner ear occurs in parallel to the natural rise of TH blood plasma levels (Deol, 1973; Knipper et al., 2000; Uziel et al., 1985).

The importance of T3 availability as a factor controlling TR-regulated transcription was clearly demonstrated in a recent study of TR\textsubscript{α1/mmt} mutant mice. In this strain, the affinity of TR\textsubscript{α1} for TH is reduced as a consequence of a point mutation (Tinnikov et al., 2002) leading to mortality within the first 3 postnatal weeks, as in Pax8\textsuperscript{-/-} mice. The pathological consequences of the condition were reversible upon compensatory T3 levels (Tinnikov et al., 2002). The importance of circulating T3 for the conversion of apo-TR\textsubscript{α1} to the holoreceptor configuration was also emphasized in studies analyzing TRα1 function in heart tissue, intestine, bone and brain (Flamant et al., 2002; Mai et al., 2004).

The role of local T3 levels, which are determined by deiodinase activity, has been shown in target tissues during amphibian metamorphosis (Becker et al., 1997; Huang et al., 1999) and rat brain development (Kaplan and Yaskoski, 1981; Obregon et al., 1991). Hearing loss in 5'-deiodinase type 2 (D2)-deficient mice (Ng et al., 2004) was only recently discussed in the context of a striking peak of D2 activity in the cochlea between P5 and P10 (Campos-Barros et al., 2000).

Although the role of deiodinase in the switch from apo-TR to holo-TR has been hypothesized (Chassande, 2003), supporting data have been unavailable until now.

The data in the present study show that the time period over which the two T3 target genes in OHCs are activated and/or modulated is brief and occurs between P6 and P10, when there is a peak in 5'-deiodinase activity (Campos-Barros et al., 2000) and a steep rise in the TH blood plasma level (Knipper et al., 2000). Local T3 levels thus may split the genes into TRα1-affected or -unaffected categories. T3 may induce a conformational change in the receptor that destabilizes its bond with co-repressors and facilitates the binding of co-activators (Lazar, 2003).

Retinoic acid, shown to be required as an additional ligand to overcome the repressive effect on Kcnq4 promoter activity in vitro (Fig. 6) should be considered as a further ligand needed for derepression. Lee and Privalsky (Lee and Privalsky, 2005) reported that TRα1 forms heterodimers with RAR, which in contrast to TR-RXR heterodimer recruit both co-activators and co-repressors. TRα1-RAR heterodimers bound to the inverted palindrome TRE-3KCNO4 in OHCs may thus recruit co-repressors until TH levels rise during early postnatal development. Although several studies underscore the importance of RAR for inner-ear development (Raz and Kelley, 1999; Romand et al., 2002), changes in retinoic acid levels during postnatal development have not been reported (Romand, 2003).

TRβ activity in the maturation of OHCs
As higher T3 levels seem to be required to release apo-TRα1 from TRE-3KCNO4, lower T3 levels may be sufficient for the recruitment of the TRβ complex to TRE\textsuperscript{Prest} (Fig. 8). Prestin expression and distribution are known to be diminished and immature in hypothyroidism (Weber et al., 2002), as well as in TRβ\textsuperscript{-/-} and TRα1\textsuperscript{+/-}/β\textsuperscript{-/-} mutants as shown in the present study. Although we cannot rule out the possibility that the influence of TH on the redistribution of prestin is indirect, this condition may be causally linked to the reduced nonlinear capacitance of OHCs observed in TRβ\textsuperscript{-/-} and TRα1\textsuperscript{+/-}/β\textsuperscript{-/-} mutants (Rusch et al., 2001).

In conclusion, the characterization of differentially regulated T3 target genes in the same cell may provide the basis for novel insight into the mechanism of subcellular TR specificity. Given the fact that TRα1 and TRβ show no preference in binding to TRE\textsuperscript{Prest} or TRE-3KCNO4, the specificity of TR binding in postnatal OHCs must be achieved by additional gene-specific differences in the upstream regions of the prestin and Kcnq4 genes. These differences influence the level of TR-associated DNA-bound transcription factors, as previously proposed (Glass and Rosenfeld, 2000). The composition of regulatory elements may then act in concert with specific local T3 levels to alter dissociation or recruitment rates of co-repressor or co-activator complexes (Fig. 8) (Hermanson et al., 2002; Kamei et al., 1996; Onate et al., 1995).

Until now human patients with mutant TRα1 have not been identified; as either the effects on a human phenotype are too mild, spontaneous miscarriages occur at embryonic or fetal stages of development, and/or the symptoms have not been attributed to defects in TRα1 (Tinnikov et al., 2002). Based on the findings reported here, the latter explanation may apply most appropriately in the case of the auditory system.

Materials and Methods
Animals and drug administration
Wistar rats, Pax8\textsuperscript{-/-} (Mansouri et al., 1998), Tshr\textsuperscript{+/-} (hypothy) (Walsh and McGee, 2001), TRα1\textsuperscript{+/-} (Wikstrom et al., 1998), TRβ\textsuperscript{+/-} (Forrest et al., 1996a), TRα1\textsuperscript{+/-}/β\textsuperscript{+/-} (Gothe et al., 1999) and TRα1\textsuperscript{+/-}/β\textsuperscript{+/-} (Tinnikov et al., 2002) mice were used (Table 1). MMI treatment of mice, thyroxin (T4) administration, and T4 and T3 plasma titer determinations were performed as described (Knipper et al., 2000; Weber et al., 2002). Animal experiments were approved and complied with all protocol requirements at the University of Tübingen.

Tissue preparation
Cochleae of untreated (control), MMI-treated (hypothyroid) and T4-treated (hypothyroid+T4) animals were prepared and cryosectioned as described (Knipper et al., 1998).

Riboprobe synthesis and in situ hybridization
Using the KCNO4-specific oligonucleotide primers rKCNO4-USP3 and rKCNO4-DSP9 (supplementary material, Table S1) a PCR fragment was amplified from rat cochlear cDNA, cloned into pCR® II TOPO Vector (Invitrogen) and sequenced. KCNO4-specific riboprobes were synthesized and in situ hybridization was performed as described (Knipper et al., 1999; Knipper et al., 2000; Weber et al., 2002).

Northern blot
Northern blots were performed as described (Knipper et al., 1998; Knipper et al., 1999). The effect of TH on mRNA levels was semi-quantitatively evaluated using mRNAs isolated from a similar number of cochleae as described (Knipper et al., 1998). To ensure the loading of similar amounts of mRNA per lane blots were probed with the housekeeping gene cyclophilin (Thellin et al., 1999).

Semi-quantitative RT-PCR
Semi-quantitative RT-PCR was performed as described (Rüttiger et al., 2006). In brief, cochlear mRNA from P12 hypothyroid and control rats was isolated using the Dynabeads mRNA Direct Kit (Dynal) and reverse-transcribed with Superscript II.
In vitro translation
Rat TRα and TRβ2 were translated in vitro from plasmids (Weber et al., 2002) using the TNT T7 coupled reticulocyte lysate system (Promega).

Electromobility-shift assay (EMSA)
EMSAss were performed as described (Weber et al., 2002). Unlabeled competitor oligonucleotides (200-fold excess) or T3 (150 nM) were preincubated with recombinant chicken TRs (Santa Cruz) for 30 minutes before adding the radiolabeled oligonucleotides.

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